Oxidative stability of phytosterols in food models and foods

Laura Soupas

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Agriculture and Forestry,
University of Helsinki, for public criticism in Auditorium XIV,
University Main Building, Unioninkatu 34, Helsinki,
on November 17th, 2006, at 12 noon.

Helsingin yliopisto
Soveltavan kemian ja mikrobiologian laitos

University of Helsinki
Department of Applied Chemistry and Microbiology

Helsinki 2006

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Back cover: Finnish rapeseed oil and natural phytosterols at their starting point

ISBN 952-10-3422-X (paperback)
ISBN 952-10-3423-8 (PDF; http://ethesis.helsinki.fi)
ISSN 0355-1180

Yliopistopaino
Helsinki 2006

Soupas, L. 2006. Oxidative stability of phytosterols in food models and foods. EKT-series 1370. University of Helsinki. Department of Applied Chemistry and Microbiology. 110 + 58 pp.

ABSTRACT

An important safety aspect to be considered when foods are enriched with phytosterols and phytostanols is the oxidative stability of these lipid compounds, i.e. their resistance to oxidation and thus to the formation of oxidation products. This study concentrated on producing scientific data to support this safety evaluation process. In the absence of an official method for analyzing of phytosterol/stanol oxidation products, we first developed a new gas chromatographic – mass spectrometric (GC-MS) method. We then investigated factors affecting these compounds' oxidative stability in lipid-based food models in order to identify critical conditions under which significant oxidation reactions may occur. Finally, the oxidative stability of phytosterols and stanols in enriched foods during processing and storage was evaluated. Enriched foods covered a range of commercially available phytosterol/stanol ingredients, different heat treatments during food processing, and different multiphase food structures.

The GC-MS method was a powerful tool for measuring the oxidative stability. Data obtained in food model studies revealed that the critical factors for the formation and distribution of the main secondary oxidation products were sterol structure, reaction temperature, reaction time, and lipid matrix composition. Under all conditions studied, phytostanols as saturated compounds were more stable than unsaturated phytosterols. In addition, esterification made phytosterols more reactive than free sterols at low temperatures, while at high temperatures the situation was the reverse. Generally, oxidation reactions were more significant at temperatures above 100°C. At lower temperatures, the significance of these reactions increased with increasing reaction time. The effect of lipid matrix composition was dependent on temperature; at temperatures above 140°C, phytosterols were more stable in an unsaturated lipid matrix, whereas below 140°C they were more stable in a saturated lipid matrix. At 140°C, phytosterols oxidized at the same rate in both matrices. Regardless of temperature, phytostanols oxidized more in an unsaturated lipid matrix.

Generally, the distribution of oxidation products seemed to be associated with the phase of overall oxidation. 7-ketophytosterols accumulated when oxidation had not yet reached the dynamic state. Once this state was attained, the major products were 5,6-epoxyphytosterols and 7-hydroxyphytosterols. The changes observed in phytostanol oxidation products were not as informative since all stanol oxides quantified represented hydroxyl compounds. The formation of these secondary oxidation products did not account for all of the phytosterol/stanol losses observed during the heating experiments, indicating the presence of dimeric, oligomeric or other oxidation products, especially when free phytosterols and stanols were heated at high temperatures.

Commercially available phytosterol/stanol ingredients were stable during such food processes as spray-drying and ultra high temperature (UHT)-type heating and subsequent long-term storage. Pan-frying, however, induced phytosterol oxidation and was classified as a rather deteriorative process. Overall, the findings indicated that although phytosterols and stanols are stable in normal food processing conditions, attention should be paid to their use in frying. Complex interactions between other food constituents also suggested that when new phytosterol-enriched foods are developed their oxidative stability must first be established. The results presented here will assist in choosing safe conditions for phytosterol/stanol enrichment.

PREFACE

The story of this thesis began in October 2000, the year I graduated. I found myself in the food chemistry laboratory analyzing phytosterols and phytosterol oxidation products – and believe me – there was fat everywhere. Ever since this fatty autumn, I have had opportunity to advance this thesis with the help of two very skillful group leaders, three researchers, and five research assistants, i.e. with the "sterol girls", at the Department of Applied Chemistry and Microbiology, Food Chemistry Division, University of Helsinki.

My deepest appreciation is due to my supervisors Professor Vieno Piironen and Docent Anna-Maija Lampi. You made this thesis possible and created a pleasant and stimulating work environment. I also gratefully acknowledge the National Technology Agency (Tekes), Fazer Group, Ingman Foods Group, Raisio Plc., Teriaka Ltd., the Finnish Graduate School Program "Applied Bioscience – Bioengineering, Food & Nutrition, Environment" (ABS), the August Johannes and Aino Tiura Foundation, and the Finnish Association of Academic Agronomists for financial support.

I am very grateful for the contributions of Laura Huikko, Suvi Kemmo and Laura Nyström to this thesis. I also thank them for the many joyful moments we have shared in our workroom, in the lab, in our homes, and wherever we have found ourselves. I have wondered if having so many Lauras in our project created a special kind of magic… I am also grateful to have had such a treasure, Laura H., as a personal research assistant. Our collaboration was effortless and perfect. Special thanks are also owed to Tanja Achrenius, Susanna Heikkinen, Saara Jouhtimäki, Marjo Toivo and Katariina Usano for joining this work for varying periods and for performing hundreds of analyses that helped me to resolve the secrets of phytosterols.

My sincere thanks are due to Professors Paresh Dutta and Francesc Guardiola for their careful work in the pre-examination of this thesis. Their constructive – and flattering – comments were invaluable.

I warmly thank Päivi Laakso from Raisio Plc. for reading the drafts of my articles and for posing good questions on many subjects. Tuomo Tupasela and Juha Lundström from MTT Agrifood Research Finland are thanked for their efforts to produce phytosterol-enriched

milks. My colleagues at the Food Chemistry Division deserve special mention for providing a congenial work atmosphere – although we occasionally had some problems with coffee milk :)

My heartfelt thanks go to my family, friends, relatives, and my childhood home “Korvala” in Sauvo for providing circumstances conducive to my forgetting this thesis for short periods. I especially want to thank my parents Mirja and Matti for their all sorts of support. Without their background in the Faculty of Agriculture and Forestry, I probably would not have started my studies there. The place called “Viikki” has meant something special to me for as long as I can remember.

Finally, I thank my husband Jarkko for everything. No days have passed without some lovely words – or flowers. This last year has been a real challenge. Simultaneously with writing the last article and this thesis, I was involved with our everlasting house renovation project. For many weeks, I was also busy with our wedding arrangements, and in the summer I tried to help in our strawberry business – in strawberry picking, packing, delivering and selling – while we also served as hosts to over 50 strawberry pickers. But here we are. Thank you, Jarkko, for the strength you have given me and for all the happy and cheerful moments that have made our days sunny.

Kytäjä, September 2006

Laura Soupas

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

I Soupas, L., Juntunen, L., Säynäjoki, S., Lampi, A.-M. and Piironen, V. 2004. GC-MS method for characterization and quantification of sitostanol oxidation products. J. Am. Oil Chem. Soc. 81: 135-141.

II Soupas, L., Juntunen, L., Lampi, A.-M. and Piironen, V. 2004. Effects of sterol structure, temperature, and lipid medium on phytosterol oxidation. J. Agric. Food Chem. 52: 6485-6491.

III Soupas, L., Huikko, L., Lampi, A.-M. and Piironen, V. 2005. Esterification affects phytosterol oxidation. Eur. J. Lipid Sci. Technol. 107: 107-118.

IV Soupas, L., Huikko, L., Lampi, A.-M. and Piironen, V. 2006. Oxidative stability of phytosterols in some food applications. Eur. Food Res. Technol. 222: 266-273.

V Soupas, L., Huikko, L., Lampi, A.-M. and Piironen, V. 2007. Pan-frying may induce phytosterol oxidation. Food Chem. 101: 286-297.

Contribution of the author to studies I-V:

I-III Laura Soupas planned the study together with the other authors. She was responsible for the GC-MS analyses performed during the study and had the main responsibility for interpreting the results. She was the main author of the paper.

IV Laura Soupas planned the study together with the other authors and performed part of the experiments. She had the main responsibility for interpreting the results and was the main author of the paper.

V Laura Soupas planned the study together with the other authors. She had the main responsibility for interpreting the results and was the main author of the paper.

LIST OF ABBREVIATIONS

ABC	adenosine triphosphate-binding cassette
AHA	American Heart Association
AMF	anhydrous milk fat
ATP	adenosine triphosphate
BHT	butylated hydroxytoluene
BSTFA	*N*,*O*-bis-(trimethylsilyl)trifluoroacetamide
CHD	coronary heart disease
CV	coefficient of variation
EI	electron ionization
EU	European Union
FDA	US Food and Drug Administration
FID	flame ionization detection
GC / GC-FID	gas chromatograph / gas chromatography with flame ionization detection
GC-MS	gas chromatography-mass spectrometry
GRAS	generally recognized as safe
HCO	hydrogenated coconut oil
HDL	high-density lipoprotein
HMDS	hexamethyldisilazane
HPLC	high-performance liquid chromatograph(y)
HPLC-MS	high-performance liquid chromatography-mass spectrometry
HPSEC	high-performance size-exclusion chromatography
ISTD	internal standard
IUPAC-IUB	International Union of Pure and Applied Chemistry and International Union of Biochemistry
KOH	potassium hydroxide
LC	liquid chromatography
LDL	low-density lipoprotein
MP	melting point
MS	mass spectrometer
MSTFA	*N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide
NCEP	US National Cholesterol Education Program
NOAEL/NOEL	no-observed-adverse-effect level / no-observed-effect level
RPKO	refined palm kernel oil
RRF	relative response factor
RRT	relative retention time
RSO	rapeseed oil
RT	room temperature
SCF	European Union Scientific Committee on Food
SIM	selected ion monitoring
SPE	solid-phase extraction
TBME/MTBE	*tert*-butylmethyl ether / methyl *tert*-butyl ether
TLC	thin-layer chromatography
TMCS	trimethylchlorosilane
TMS	trimethylsilyl
UHT	ultra high temperature
WHO	World Health Organization

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ORIGINAL PUBLICATIONS I-V

1 INTRODUCTION

Cardiovascular diseases, especially coronary heart disease (CHD), are the leading causes of death in the world today. According to the World Health Organization (WHO), 16.7 million cardiovascular disease deaths occurred in 2002, i.e. one-third of the total global deaths. Of people dying of CHD, 80-90% have one or more major lifestyle risk factors, including high blood pressure, high cholesterol, obesity, and smoking (WHO, 2003).

High cholesterol was identified long ago as one of the risk factors of CHD. Two Russian physiologists, Anitschkow and Chalatow, showed in 1913 that cholesterol-feeding induced atheroma in rabbits (Anitschkow and Chalatow, 1913). Some 40 years later, phytosterols were found to lower serum cholesterol first in animals (Peterson, 1951) and then in humans (Pollak, 1953). When Pollak (1953) finished his article by writing that "this preliminary report should open a new avenue of research", he was more than aware of the future. His conclusion that ingestion of sitosterol in proper amounts prevents intestinal resorption of cholesterol was undoubtedly one of the key steps towards today's growing markets of cholesterol-lowering phytosterol-enriched functional foods.

Initially, phytosterols were used as pharmacological agents (Kritchevsky and Chen, 2005). For a long time, however, these compounds were considered to be of little practical interest mainly due to their poor solubility and thus difficulties in their administration (Miettinen and Gylling, 1999; Moreau et al., 2002). A real breakthrough was the recognition that these cholesterol-lowering compounds could be used as part of the normal human diet. The train of events leading to this recognition started in 1986 with the observation that sitostanol administration at a dose of 1.5 g/d resulted in significant lowering of human total cholesterol (Heinemann et al., 1986). This finding of sitostanol being effective at relatively small doses led first to the development of fat-soluble phytostanol esters (Vanhanen et al., 1994; Gylling and Miettinen, 2000) and finally to the launch of the first commercial phytostanol-enriched food application, Benecol® margarine (Raisio Plc. Raisio, Finland), in Finland, in November 1995.

Several other formulations, the first including esterification of phytosterols (Moreau et al., 2002), have subsequently been developed to reduce technological limitations of phytosterols and stanols and to increase their potential for incorporation into foods (Corliss et al., 2000; Akashe and Miller, 2001; Christiansen et al., 2001; SCF, 2003c; Engel and Knorr, 2004). In addition, as alternatives to the first commercial food application, margarine, other food types, including low-fat and even nonfat products, have been introduced to the market.

A noteworthy scientific basis for the use of Benecol® was the study conducted by Miettinen et al. (1995); a one-year study that established the effectiveness of consumption of phytostanol ester-

enriched margarine in lowering cholesterol levels (Miettinen et al., 1995). Since then, dozens of studies have been carried out on the effects of dietary phytosterols and stanols on lowering cholesterol in a wide variety of subjects, leading to the conclusion that, in general, a reduction of 10-15% in serum low-density lipoprotein (LDL) cholesterol can be achieved by a phytosterol or stanol intake of 1.5-3 g/d (Normén et al., 2004).

In the evaluation of functional foods, another key aspect, besides efficacy, is safety (Palou et al., 2003). Phytosterols and stanols have been used for lowering serum cholesterol levels for half a century now, and, thus far, no marked adverse effects have been reported (Katan et al., 2003; Kritchevsky, 2004; Berger et al., 2004, Gylling and Miettinen, 2005). The US Food and Drug Administration (FDA) has granted GRAS (generally recognized as safe) status to many phytosterol/stanol ingredients (FDA, 2000a,b,c; 2001; 2003; 2005; 2006a,b) and the European Union Scientific Committee on Food (SCF) has thoroughly reviewed the safety of phytosterols, stanols, and their esters and sanctioned them for use in a variety of foods (SCF, 2000; 2003a,b,c). However, hardly anything is known about the oxidative stability of phytosterols and stanols in foods, although the formation of oxidation products has been among the important safety questions of phytosterol-enriched products raised by European Union (EU) member states (SCF, 2000).

Concern regarding oxidative stability is warranted since sterols, as unsaturated lipid compounds, are susceptible to oxidation (Labuza, 1971; Maerker, 1987; Dutta, 2004). Although biological effects of phytosterol oxidation products have been studied little, research on structurally related cholesterol oxidation products has revealed that sterol oxides may be linked to a series of human diseases by exerting a deleterious effect on lipid metabolism and cell function (Osada, 2002). Cytotoxicity studies have indicated similar patterns of toxicity for cholesterol and sitosterol oxides (Adcox et al., 2001; Maguire et al., 2003; Ryan et al., 2005).

Since evidence exists that phytosterol oxides may have adverse effects on human health, their contents in enriched foods and the effect of food processing and storage on their formation should be investigated. To date, however, the presence of phytosterol oxides has mainly been determined in some vegetable oils (Lambelet et al., 2003; Bortolomeazzi et al., 2003; Zhang et al., 2005a) and fried potato products (Dutta and Appelqvist 1996a; Dutta and Appelqvist, 1997; Dutta 1997). No systematic research has been carried out on phytostanol oxidation. Considering that the intake of phytosterols from enriched food products is 8- to 12-fold that of the natural daily intake (SCF, 2000), studies on phytosterol oxide contents in enriched foods providing data on possible intakes of these oxides should be undertaken. The main reason for the lack of phytosterol oxidation studies probably lies in the absence of commercial phytosterol oxide standards, and thus, in the absence of official methods for oxide determination. Efforts are therefore required to find appropriate ways of establishing oxide contents in foods irrespective of possible low amounts or difficult food matrices.

This study concentrated on producing scientific data to support the safety evaluation process of phytosterol- and phytostanol-enriched foods with regard to phytosterol/stanol oxidation. In the absence of an official method for evaluating phytosterol/stanol oxidation products, we first developed a new gas chromatographic-mass spectrometric (GC-MS) method for their analysis. We then examined factors affecting their oxidative stability in order to identify critical conditions under which significant oxidation reactions may occur. Finally, oxidative stability of phytosterols and stanols in enriched foods during processing and storage was evaluated.

In this thesis, "phytosterol" is used as a generic term, including both plant-derived Δ^5-phytosterols and saturated phytostanols. For the sake of clarity, however, these compounds are mainly discussed separately. Furthermore, phytosterol esters and phytostanol esters here refer to their fatty acid esters.

2 REVIEW OF THE LITERATURE

2.1 Phytosterols as natural and novel food components

2.1.1 Structures and natural sources of phytosterols

Phytosterols (plant sterols) are natural constituents of plants and are part of the broad group of isoprenoids. Phytosterols have many essential functions in plant cells; they regulate the fluidity and permeability of cell membranes and act as biogenetic precursors of compounds involved in plant growth, e.g. brassinosteroids. In addition, they are substrates for the synthesis of numerous secondary plant metabolites such as glycoalkaloids and saponins (Hartmann, 1998).

Both on structural and biosynthetic grounds, sterols can be divided into 4-desmethylsterols, 4α-monomethylsterols, and 4,4-dimethylsterols. 4α-monomethylsterols and 4,4-dimethylsterols are biosynthetic intermediates leading to the end-product, 4-desmethylsterols. The term 4-desmethylsterol covers compounds with no methyl substitution at C-4 and is synonymous with the original name "sterol" (Akihisa et al., 1991; Goad, 1991). By 1991, in excess of 250 sterols and related compounds had been separated in plants, over 100 of which were 4-desmethylsterols, i.e. these constitute the dominant fraction (Akihisa et al., 1991).

4-desmethylsterols include all of the common phytosterols with a 28- or 29-carbon skeleton, but also cholesterol with a 27-carbon skeleton (Moreau et al., 2002). 4-desmethylsterols possess a skeleton of cyclopenta[*a*]phenanthrene, with a hydroxyl group at C-3, methyl groups at C-10 and C-13, and an alkyl side chain with 8-10 carbons at C-17 (Moss, 1989; Goad, 1991). The structural similarity between phytosterols and mammalian cholesterol is striking; they mainly differ by a methyl or an ethyl group at the C-24 position in the side chain (Figure 1). Most phytosterols as well as cholesterol have a double bond between C-5 and C-6 in the tetracyclic ring system and thus are called Δ^5-sterols. Another group of common phytosterols are Δ^7-sterols, with a double bond between C-7 and C-8, and $\Delta^{5,22}$-sterols with double bonds between C-5 and C-6 and also between C-22 and C-23. Saturated phytosterols with no double bonds in their structure are called phytostanols (Figure 1). The main phytosterol encountered in higher plants is sitosterol. It is often accompanied by its 22-dehydroanalog, stigmasterol. Campesterol also occurs widely in plants (Akihisa et al., 1991; Goad, 1991; Moreau et al., 2002) (Figure 1).

In animals, body cholesterol occurs mainly in free form (as alcohol) and to a small extent as long chain fatty acid esters (Ostlund, 2002). However, phytosterols have a more diverse variety of derivatives in plants; they occur in free form but also in conjugated forms, i.e. as fatty acid esters, steryl glycosides, or acylated steryl glycosides (Wojciechowski, 1991). Corn and rice seeds and some other grains also contain phytosteryl hydroxycinnamic-acid esters, in which sterol is esterified

to ferulic or *p*-coumaric acid (Moreau et al., 2002; Moreau, 2005). In free form, the hydroxyl group at the C-3 position in the tetracyclic ring structure is underivatized (Figure 1), whereas in conjugated forms, the hydroxyl group is covalently bound to other constituents (Moreau, 2005) (Figure 2).

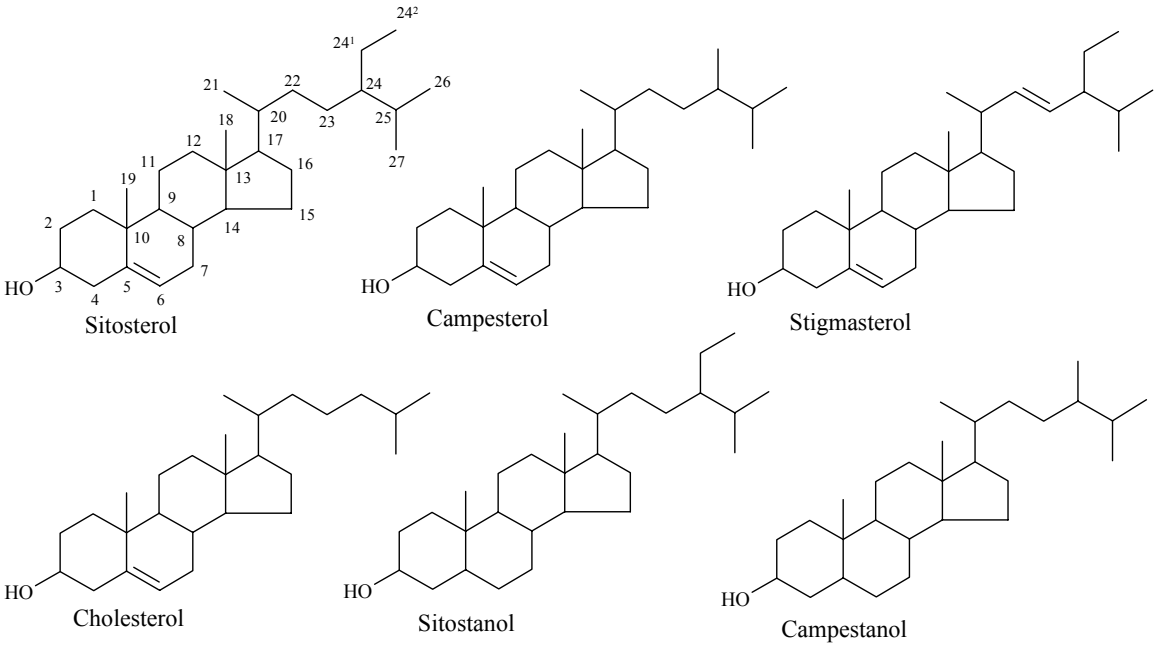

Figure 1. Structures of common phytosterols, phytostanols, and cholesterol. Numbering is according to the International Union of Pure and Applied Chemistry and International Union of Biochemistry (IUPAC-IUB) (1989) recommendations (Moss, 1989).

Phytosterols are not synthesized by animals or humans, unlike cholesterol, but as plant constituents are natural components in the human diet (Ratnayake and Vavasour, 2004). Dietary intakes of phytosterols from natural sources are estimated to range between 150 and 440 mg/d in various populations (De Vries et al., 1997; Schothorst and Jekel, 1999; Ostlund, 2002). However, in vegetarians, the intake of phytosterols can be much higher (Vuoristo and Miettinen, 1994; Ling and Jones, 1995; Piironen et al., 2000). A recent study of phytosterol intakes in Finland showed a total phytosterol intake of 305 mg/d for men and 237 mg/d for women. These intakes comprised 24 mg/d and 17 mg/d of stanols (sitostanol and campestanol), respectively (Valsta et al., 2004). In general, dietary intake of phytostanols seems to be about 10% of phytosterol intake (Ostlund, 2002). The phytosterol:cholesterol ratio of the diet normally lies between 0.5 and 1.0, but has recently been shown to exceed 1.0 (Valsta et al., 2004).

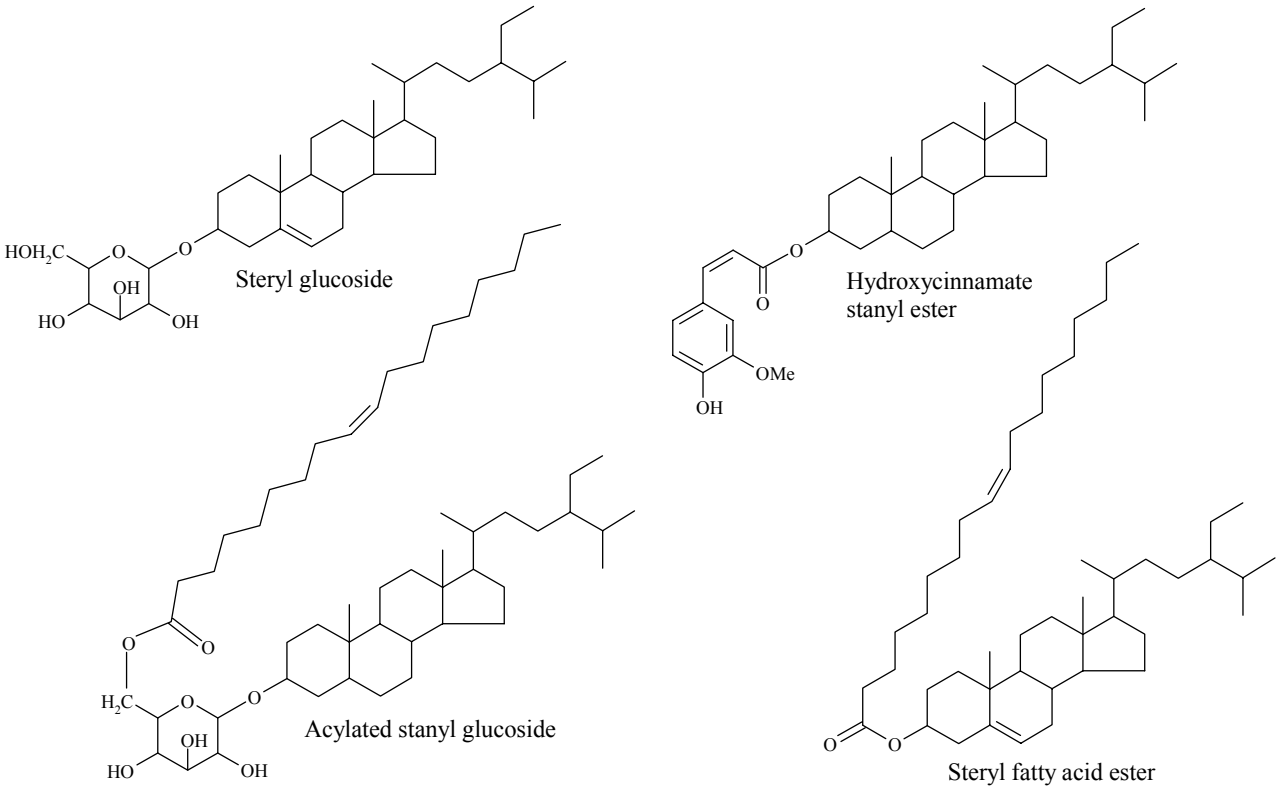

Figure 2. Examples of phytosteryl conjugates.

In general, the main sources of phytosterols in the human diet are vegetable oils, vegetable oil-based food products, cereal grains, cereal-based foods, and nuts. According to a recent study in Finland, cereal and cereal products, especially rye, were the major sources for phytosterols, followed by margarine (Valsta et al., 2004). Phytosterol content of crude vegetable oils lies between 70 and 1100 mg/100 g of oil, with rapeseed and corn oils being the richest sources, and olive and palm oils the poorest (Piironen and Lampi, 2004). Some special oils, like rice bran oil and wheat germ oil, can contain phytosterols in amounts up to 3200 mg/100 g of oil (Piironen et al., 2000). The most unique oil seems to be corn fiber oil, which contains phytosterols in quantities of about 10 000 mg/100 g of oil (Moreau, 2005). Among cereals, the richest sources of phytosterols are corn (65-120 mg/100 g) and rye (70-110 mg/100 g), while wheat, barley, and oats contain 35-80 mg/100 g (Piironen and Lampi, 2004). Different nuts contain phytosterols in the range of 30-220 mg/100 g (Piironen et al., 2000).

Despite their high moisture content, the contribution of vegetables, fruits, and berries in phytosterol intake has also been shown to be significant (Piironen et al., 2003; Valsta et al., 2004). On a fresh weight basis, phytosterol contents of fresh vegetables range from 5 to 40 mg/100 g, the highest contents measured in broccoli, Brussels sprouts, cauliflower, and dill. In fresh fruits, phytosterol contents lie within the range of 10-20 mg/100 g fresh weight, and in fresh berries 6-30 mg/100 g

fresh weight. Wild berries, like lingonberry and blueberry, are more phytosterol-rich than cultivated berries such as blackcurrant, redcurrant, and strawberry (Piironen et al., 2003).

2.1.2 Dietary phytosterols as cholesterol-lowering compounds

The history of using phytosterols for cholesterol-lowering purposes started in the early 1950s, when Peterson (1951) showed that the addition of soybean sterols in the presence of cottonseed oil and cholesterol to the chicks' diet resulted in lower serum cholesterol levels than a diet of cholesterol and cottonseed oil mixture alone. Short after, Pollak (1953) demonstrated that ingestion of sitosterol prevented intestinal absorption of cholesterol in humans, resulting in a lowering of serum cholesterol. Since then, phytosterols have been used for treatment of hypercholesterolemia, the first commercial product being Cytellin (from the pharmaceutical company Eli-Lilly Co., Indianapolis, IN, USA), which was marketed from the late 1950s until 1982 (Moreau et al., 2002; Trautwein et al., 2003). Due to the low efficacy of crystalline phytosterols, very high doses (up to 50 g/d) were used, but the cholesterol-lowering effects were nevertheless observed to be variable (Miettinen and Gylling 1999; Moreau et al., 2002; Kritchevsky and Chen, 2005). These problems and the availability of "statin" drugs led to a diminished interest in phytosterols (Miettinen and Gylling, 1999; Moreau et al., 2002) although their physical properties were improved by esterification with fatty acids already in the 1970s (Mattson et al., 1977).

Also in the 1970s, phytostanols were shown to reduce serum cholesterol in animals (Sugano et al., 1977; Ikeda et al., 1981). The first study of the use of phytostanols for serum cholesterol-lowering purposes in humans was conducted by Heinemann et al. (1986). They administered sitostanol at a dose of 1.5 g/d for 4 weeks to 6 patients with hypercholesterolemia and observed that total cholesterol was reduced by 10% and 15% after 3 and 4 weeks, respectively. Application of phytostanols in human studies and observations of their cholesterol-lowering properties at relatively small doses led first to the development of fat-soluble phytostanol esters (Vanhanen et al., 1994, Gylling and Miettinen, 2000, Piironen et al., 2000) and later to the launch of the first commercial phytostanol ester-enriched food application, Benecol® margarine (Raisio Plc., Raisio, Finland), in Finland, in November 1995 (Wester, 2000; Salo et al., 2005). A strong scientific basis for the use of Benecol® was a study of Miettinen et al. (1995), a one-year study that established that the consumption of sitostanol ester-enriched margarine (1.8 or 2.6 g of sitostanol per day) resulted in 10.2% of the mean one-year reduction in serum cholesterol (Miettinen et al., 1995).

The use of fat-soluble phytostanol esters led also to renewed interest in using phytosterols for cholesterol-lowering purposes, and in 1999, Unilever's Take control® (known also as Becel or Flora pro.activ™), a phytosterol ester-enriched margarine, came onto the market in USA (Moreau et al., 2002). The cholesterol-lowering effect of phytosterol ester-enriched margarine was shown to be similar to that of phytostanol ester-enriched margarine. Serum total and LDL-cholesterol levels

were reduced by 8-13% with consumption of 1.5-3.3 g/d of phytosterols during four 3.5-week periods (Weststrate and Meijer, 1998). New formulations have subsequently also enabled the use of free phytosterols and stanols in cholesterol management. Christiansen et al. (2001) demonstrated the efficacy of microcrystalline free phytosterols; serum total and LDL-cholesterol levels were reduced by 7.5-11.6% by consuming phytosterol-enriched margarine (1.5 or 3.0 g of phytosterols per day) during a 6-week run-in and a 6-month experimental period.

To date, dozens of studies have been conducted on the effects of dietary phytosterols and stanols on lowering cholesterol in a wide variety of subjects. Katan et al. (2003) summarized the results of 41 randomized trials comparing the effect of mainly esterified phytosterols and stanols added to margarine, mayonnaise, olive oil, or butter. Their meta-analysis showed that with a daily intake of 2 g of phytosterols or stanols a reduction of 10% in LDL-cholesterol was achieved. The subjects in these trials mainly had rather high cholesterol levels. Recently, Normén et al. (2004) gave an overview of the results of 57 clinical studies of serum cholesterol-lowering effects of free and esterified phytosterols and stanols. In these studies, spreads, dressings, ground beef, yoghurt, mayonnaise, lemonade, pudding, chocolate, and capsules were used as food vehicles. An average daily dose of 2.8 g/d of phytosterols/stanols led to an overall LDL-cholesterol reduction of 10.9% in normo- and mildly hypercholesterolemic subjects.

In general, a reduction of 10-15% in serum LDL-cholesterol levels can be achieved by a phytosterol or stanol intake of 1.5-3 g/d and in the presence of any kind of diet (Normén et al., 2004). The effect can be seen after 2-3 weeks of initiation of treatment (Jones et al., 1997; Hallikainen and Uusitupa, 1999) and has been observed in normo- and hypercholesterolemic subjects (adults and children) as well as in subjects with familial hypercholesterolemia, type II diabetic hypercholesterolemic patients, and type II diabetic subjects in conjunction with statin therapy (Berger et al., 2004; Gylling and Miettinen, 2005). Doses higher than 3 g/d do not yield any further significant improvements (Vanhanen et al., 1994; Miettinen and Gylling, 2004; Normén et al., 2004), while doses lower than 1 g/d are too small for practical serum cholesterol-lowering in hypercholesterolemic subjects (Miettinen and Vanhanen, 1994; Vanhanen et al., 1994; Hallikainen et al., 2000). To sum up, it seems to be generally accepted that the optimum effect can be obtained with about 2 g of phytosterols or stanols per day (Piironen et al., 2000; Wester, 2000; Ostlund et al., 2002; Salo et al., 2002; Gylling and Miettinen, 2005), and this dose is recommended in the latest published US National Cholesterol Education Program (NCEP) guidelines (Anonymous, 2001). Noteworthy, however, is that after cessation of phytosterol administration, serum cholesterol levels return to baseline within two weeks (Heinemann et al., 1986; Jones et al., 1997)

Since the very first studies, phytostanols have been speculated to be more effective in lowering cholesterol than phytosterols (Sugano et al., 1977; Ikeda et al. 1981). This finding was not, however, supported by the meta-analysis of 41 trials by Katan et al. (2003), which showed almost

identical absolute reductions in total and LDL-cholesterol for phytosterols and stanols. In summarizing the results of five studies comparing the effects of phytosterol and stanol esters (published in 1998-2002), Moreau et al. (2002) also concluded that phytosterols and stanols are roughly equivalent in their cholesterol-lowering efficacy. However, in a recent study, phytosterol and stanol esters were again compared, and data suggested that phytostanol esters maintain their efficacy both short- and long-term, while the effect of phytosterol esters is less marked long-term. The authors suggested that high intakes of phytosterols suppress bile acid synthesis. This lowers the elimination of cholesterol from the body as bile acids, thus preventing a reduction in LDL-cholesterol (O'Neill et al., 2005). The same effect was earlier suggested by Miettinen and Gylling (2004), who compared studies on long-term use of phytosterol and stanol esters and found a lack of efficacy in phytosterol ester.

Possible differences in cholesterol-lowering ability of free and esterified phytosterols and stanols have also been studied. Nestel et al. (2001) reported that in their 12-week test period no statistically significant differences were observed between the effects of phytosterol esters and free phytostanols. Jones et al. (1999) evaluated the effect of free tall oil phytosterols provided over a 30-day period and found comparable LDL-cholesterol reductions to those achieved with phytostanol esters over longer periods. Christiansen et al. (2001) showed that microcrystalline free phytosterols were as effective as their ester forms when consumed during a 6-week run-in and a 6-month experimental period. Thus, properly formulated free phytosterols and stanols may be as effective as phytostanol and sterol esters (Moreau et al., 2002).

In addition to the variables introduced above, there are other factors that might influence the efficacy phytosterols and stanols. Subjects of active discussion are the frequency of administration and the food vehicles into which the phytosterols and stanols are incorporated. These topics are introduced later in this section and in section 2.1.3.

Mechanism of action

Although many studies have been conducted to resolve the mechanisms of action by which phytosterols lower serum cholesterol, the molecular actions are not fully understood. The main physiological response to ingestion of phytosterols is known to be reduced intestinal absorption of both dietary and endogenously produced cholesterol without, however, any decrease in the levels of high-density lipoprotein (HDL) -cholesterol or triglycerides (Moreau et al., 2002, Ostlund et al., 2002). This interference with absorption is probably related to the similarity in the chemical structures of phytosterols, stanols, and cholesterol (Salo et al., 2002; Plat and Mensink, 2005).

Possible mechanisms by which the interference with cholesterol absorption occurs may be the reducing or blocking of cholesterol ester hydrolysis performed by lipases and cholesterol esterases,

the co-precipitation of cholesterol and phytosterols and stanols to form nonabsorbable mixed crystals, and the competition for incorporation into dietary mixed micelles (the "packages" that deliver mixtures of lipids for absorption into enterocytes), thus limiting the amount of cholesterol available for absorption (Trautwein et al., 2003). Although good experimental support exists for "the theory of mixed micelles" (Ostlund, 2002; Trautwein et al., 2003), the other above-mentioned mechanisms may also be valid since phytosterols and stanols are absorbed under the same conditions as cholesterol. Like cholesterol, dietary phytosterols and stanols have to solubilize into the mixed micelles to be able to enter the enterocytes, and if they are esterified they must first be hydrolyzed in the upper small intestine, again similar to cholesterol esters (Trautwein et al., 2003; Miettinen and Gylling, 2004).

Current studies focus on the effects at the epithelial cell level, suggesting that there is an additional longer-lasting process in which phytosterols and stanols actively influence cellular cholesterol metabolism within intestinal enterocytes (Normén et al., 2004; Plat and Mensink, 2005; Thompson and Grundy, 2005). This hypothesis was made based on the result that daily consumption frequency (a single dose or 3 divided doses) did not determine the cholesterol-lowering efficacy of phytostanol esters in margarine, i.e. there was no need to consume phytostanols simultaneously with dietary cholesterol to achieve the cholesterol-lowering effect (Plat et al., 2000). Similar behavior was later established for phytosterols in a ground beef matrix (Matvienko et al., 2002), for phytostanol esters in pasta and meat products (Salo et al., 2005), and for phytosterol esters in yoghurt drinks (Doornbos et al., 2006).

In enterocytes, phytosterols and stanols potentially increase the expression of ABC (adenosine triphosphate (ATP) binding cassette) transporters, integral membrane proteins that use the energy generated from ATP hydrolysis for transportation of a substrate across the membrane. Of these, ABCG5, ABCG8, and probably ABCA1 are identified as specific sterol transporters. When dietary phytosterols or stanols enter the enterocytes via micellar transport, they are assumed to be directly transported back to the intestinal lumen by the ABCG5-ABCG8 complex, thus keeping their absorption low. This complex, stimulated by phytosterols and stanols, may also promote the efflux of cholesterol, thereby regulating the total sterol absorption (Schmitz et al., 2001; Gylling and Miettinen, 2005). The suggestion of the incorporation of the ABCG5-ABCG8 complex in transport of phytosterols and stanols is based on the finding that in phytosterolemia (or sitosterolemia, a rare human genetic disorder affecting phytosterol metabolism) the high absorption of phytosterols was due to the deficiency of these transporters (Schmitz et al., 2001; Yu et al., 2002; Gylling and Miettinen, 2005). However, other transporters are also thought to participate in sterol absorption mechanisms, a recent finding being the Niemann-Pick C1-Like 1 transporter (von Bergmann et al., 2005; Gylling and Miettinen, 2005). Mechanisms by which phytosterols and stanols affect cholesterol transportation over the enterocyte membrane are still largely open and why phytosterols

are taken up by the enterocytes and then resecreted back into the intestine remains an open question (von Bergmann et al., 2005).

The above-mentioned theory is supported by a recent study showing that mixed micelles enriched with sitostanol or with cholesterol and sitostanol increased intestinal expression of ABCA1 in Caco-2 cells (Plat and Mensink, 2002). In subsequent animal studies, metabolites of phytosterols have been observed to activate liver X receptors, which, in turn, activate the expression of ABCG5 and ABCG8 transporters. However, the metabolic conversion of dietary phytosterols has not been elucidated (Kaneko et al., 2003). The potentially important role of ABCG5 and ABCG8 transporters in sterol trafficking has also been tested by disrupting *Abcg5* and *Abcg8* in mice. This led to a 30-fold increase in serum sitosterol and extremely low biliary cholesterol concentrations (Yu et al., 2002).

Relevance of lowering cholesterol by dietary phytosterols and stanols

Dietary tools for lowering cholesterol, by means of phytosterols and stanols have been available for over ten years now. As introduced above, phytosterols and stanols have the potential to play an important role in the control of cholesterol levels, both in the general population and in additive way in statin treatment of patients with CHD (Katan et al., 2003; Miettinen and Gylling, 2004; Normén et al, 2004). Considered from population level, the reduction of about 10% in LDL-cholesterol is significant and expected to lead to a 12-20% reduction in short-term CHD risk (Katan et al., 2003). Over a longer period, the risk reduction is predicted to be 20% (Katan et al., 2003) or even 25% (Law, 2000). Noteworthy, however, is that no data exist on the effect of phytosterol/stanol consumption on CHD development (Normén et al., 2004).

Recently, NCEP Adult Treatment Panel III identified LDL-cholesterol as a major cause of CHD and set this compound as the primary target of cholesterol-lowering therapies. Furthermore, they established dietary therapy as the initial cornerstone of strategies to lower LDL-cholesterol and added the use of phytosterols and stanols (2 g/d) as part of therapeutic lifestyle changes (Anonymous, 2001). The American Heart Association (AHA) has similar recommendations to those of NCEP, although they classify phytosterols and stanols as issues that merit further research (Anonymous, 2000a). These guidelines together with recommendations from many different national associations have thus identified phytosterols and stanols as important dietary options (Normén et al., 2004) or even “the most powerful components of maximal dietary therapy” (Grundy, 2005). Noteworthy also is that in September 2000, the FDA issued a rare interim final rule authorizing the use of the health claim that phytosterol and stanol esters (consumed in foods low in saturated fat and cholesterol) may reduce the risk of CHDs by lowering blood cholesterol levels (Anonymous, 2000b).

2.1.3 Incorporation of phytosterols into foods

Research leading to the launch of Benecol® margarine was an enormous breakthrough in commercial-scale production of phytostanol/sterol-enriched foods and in the recognition that phytosterols and stanols can be part of the normal diet. These compounds were long considered to be of little practical importance mainly due to their poor solubility and thus difficulties in their administration (Miettinen and Gylling, 1999; Moreau et al., 2002). Incorporation of phytosterols and stanols into food products to make them available for consumers has been challenging.

To date, commercial phytosterols and stanols have been by-products of vegetable oil refining and the wood processing industry (SCF, 2000; 2003a,b; Salo et al., 2002; Moreau, 2004). Vegetable oil phytosterols can be isolated from soybean, corn, sunflower and rapeseed (canola) oils as well as from palm, cottonseed, and peanut oils during the deodorization step of the refining process (SCF, 2000; 2003a,b; Salo et al., 2002; Moreau, 2004). Deodorization leads to a distillate that contains 15-30% phytosterols (Moreau, 2004). The sterol fraction is further purified from solvents by crystallization (Salo et al., 2002; SCF, 2003a).

In the wood processing industry, phytosterols are derived from coniferous trees. In the case of wood-based sterols, wood extractives are at first brought into alkaline aqueous solution, and phytosterols are then recovered in a fatty and resin acid soap fraction with many other neutral substances. Finally, phytosterols are separated from the soap by liquid extraction and then from the other neutral components by crystallization. The soap fraction is further processed by a sequence of distillations. Phytosterols can also be recovered from the tall oil pitch (tall oil sterols), which is a fractionation residue of the first distillation. Phytosterols are extracted from this tall oil pitch and then crystallized (Salo et al., 2002; SCF, 2003a,b). Phytosterol contents in tall oil soaps are 3-5% and in tall oil pitch around 10% (Salo et al., 2002).

The compositions of wood and vegetable oil-derived phytosterols and stanol mixtures differ from each other. For instance, the relative contents of sitosterol, campesterol, stigmasterol, and sitostanol have been found to be 72%, 8.2%, 0.3%, and 15.3% in wood-derived and 45%, 26.8%, 19.3%, and 2.1% in vegetable oil-derived sterols, respectively (Salo et al., 2002). Differences in compositions have previously been assumed to affect the cholesterol-lowering action of these sterol mixtures. However, the results of three separate studies have shown that there is no significant difference in the LDL-lowering effect of, at least, sitostanol ester-rich versus campestanol ester-rich mixtures (Salo et al., 2002; Thompson and Grundy, 2005).

Purified phytosterols and stanols form stable crystals and possess limited solubility in both water and oil. At room temperature, the solubility of free phytosterols has been reported to be 0.01% in water (Engel and Knorr, 2004), 3.5-4.0% (w/w) in oil, and 1.5-2.0% (w/w) in oil when water is

present (Christiansen et al., 2002). The solubility of free phytostanols in oils and fats is <1% (w/w) at room temperature (Wester, 2000). Due to this poor solubility, free phytosterols may take several days to dissolve in bile salt solutions (Armstrong and Carey, 1987; Ostlund et al., 1999). To make them more soluble in the emulsified fat phase of food digest, thus promoting their efficacy lowering cholesterol, they need to be formulated.

Phytosterol and stanol esters

The esterifications of phytostanols and later phytosterols with fatty acids were the first successful formulations (Wester, 2000, Moreau et al., 2002). Esterification changes crystalline phytosterols and stanols with melting points of 140-170°C (Anonymous, 1996) to fat-like substances with a solubility of 20-30% in oil (Jandacek et al., 1977; Mattson et al., 1982). Phytosterol and stanol esters are very similar in behavior to vegetable oils, thus facilitating their incorporation into the fat phase of foods (Wester, 2000; Trautwein et al., 2003). Furthermore, as esterified phytosterols and stanols are fat-soluble, they can be evenly distributed into the fat phase of food digest (Salo et al., 2002).

Esterification is made through transesterification with food-grade fatty acids from different vegetable oils and free phytosterols and stanols. The reaction is carried out at an elevated temperature under mild vacuum and by using a catalyst (SCF, 2003c; Salo et al., 2005). When this reaction is completed, the washing to inactivate the catalyst, bleaching, and deodorization take place similar to edible oil processing (Salo et al., 2005). Esterification, when using rapeseed oil fatty acids, leads to a molecule in which ~60% comprises the sterol/stanol part (Wester, 2000). Phytostanol esters differ from phytosterol esters in that phytostanols must first be produced by hydrogenation of phytosterols in the presence of a catalyst (Salo et al., 2005).

As summarized by Thompson and Grundy (2005), almost all of the published phytostanol ester studies have used stanols esterified with rapeseed oil (RSO) fatty acids, the main resulting esters thus being phytostanol oleates (60%). Indeed, esterification of RSO fatty acids is meaningful since it leads to a stanol ester with similar melting properties to traditional fat fractions in fat blends and thus can replace part of the triglyceride fat in spreads. Noteworthy, moreover, is that the physical properties of phytostanol esters, as well as phytosterol esters, can largely be tailored by varying the fatty acid composition. Depending on the food matrix to be enriched, esters can be tailored to be liquid or solid at ambient temperature, and, furthermore, they can be modified to be nutritionally more beneficial than the fat they replace (Wester 2000; Salo et al., 2005). A new approach to fatty acid ester applications is phytosterols esterified with fish oil enriched in long-chain (n-3) polyunsaturated fatty acids (Ewart et al., 2001). This approach is, however, in the developmental stage (Moreau, 2004).

The fatty acid composition of phytosterol and stanol esters can be varied without any significant decrease in cholesterol-lowering ability. This was already reported in the 1970s, when phytosterols esterified with short (acetate), medium (decanoate), and long (oleate) -chain fatty acids were observed to be equally effective (Mattson et al., 1977). Gylling and Miettinen (1999) later showed that phytostanols esterified with butter fatty acids and dissolved in butter were as effective in lowering LDL-cholesterol as phytostanols esterified with RSO fatty acids and dissolved in margarine.

Formulations for free phytosterols and stanols

To date, several formulation methods besides esterification have been developed to reduce technological limitations of free phytosterols and stanols and to increase their potential for incorporation into foods. Formulation of these compounds is challenging since their efficacy seems to depend on their physical form, their solubility in the food matrix, and the fat content of the food (Doornbos et al., 2006).

One new approach to produce an effective form of phytosterols is to formulate a microcrystalline suspension. This kind of suspension is prepared by heating the mixture of phytosterols and oil/fat to 100-110°C and then cooling it to 90°C. Water of the same temperature is then added, and the suspension formed is stirred until it reaches room temperature (RT). Using this crystallizing method, up to 30% of phytosterols can be added to fats and oils without any additives such as emulsifying agents. In the resulting suspension, phytosterols exist in both dissolved and microcrystalline forms (Christiansen et al., 2001, 2002). This kind of microcrystalline phytosterol suspension was commercialized by Teriaka Ltd. (Finland) under the brand name Diminicol®.

Other formulations include, for instance, Multibene® (Pharmaconsult Ltd., Finland), a combination of phytosterols and mineral salts, in which phytosterols are micronized in an air jet micronizer leading to a particle size of less than 20 microns (SCF, 2003c). The bioavailability of free phytosterols and stanols can also be increased with incorporation into liposomes (Engel and Knorr, 2004). The use of mesophase-stabilized compositions for delivery of sterols and stanols in food products (Akashe and Miller, 2001) and the phytosterol-protein complex (Corliss et al., 2000) have been patented by Kraft Foods, Inc. (USA) and Monsanto Co. (USA), respectively, as methods to formulating free phytosterols and stanols. In the phytosterol-protein complex, free phytosterols are first dissolved in edible oil, and then protein, functioning as a carrier, is added. The resulting complex is ready to be used or can be dried (Corliss et al., 2000). Mesophase-stabilized compositions include both emulsions and dispersions prepared using free phytosterols, emulsifiers, and both oil and aqueous phase (emulsion) or only aqueous phase (dispersion). The resulting compositions form liquid crystalline phases of both hydrophobic and hydrophilic character (Akashe and Miller, 2001).

A promising formulation seems to be the emulsification of free phytosterols or stanols with lecithin. Lecithin disperses in water and can form water-soluble micelles that include phytosterols/stanols. This formulation is compatible with nonfat foods, requires only small amounts of phospholipids, and is speculated to be more effective in solubilizing phytosterols and stanols than triacylglycerols (Ostlund et al., 1999; Spilburg et al., 2003). Phytosterols can also be dissolved in diacylglycerol, and their solubility in diacylglycerols is greater than in triacylglycerols, i.e. 6.0% and 1.3%, respectively. Diacylglycerols are natural minor components in vegetable oils and fats and are currently used as emulsifiers in foods (Meguro et al., 2001). At the moment, phytosterol-enriched diacylglycerol oil is marketed in Japan under the brand name Econa® by ADM Kao LLC, a joint venture of Archer Daniels Midland Company and Kao Corporation of Japan (Salo et al., 2002). No plans have, however, been revealed concerning the launch of phytosterol-enriched diacylglycerol oil in, for instance, USA (Moreau, 2004).

Aspects to food vehicles

Spreads (margarines) were the first commercial applications of phytosterol/stanol-enriched foods and are still the most common enriched food types (Salo et al., 2002). In fact, already in the 1970s, in the studies of Mattson and coworkers, dietary fat was considered a good vehicle for phytosterols since it is also a carrier of cholesterol (Mattson et al., 1977; 1982). Thus far, most clinical trials carried out have used phytosterol- or stanol-enriched spreads (Berger et al., 2004).

Incorporation of phytosterols and stanols into high-fat foods is, however, contrary to the current dietary recommendations for a heart-healthy lifestyle (St-Onge and Jones, 2003). Furthermore, some food cultures do not include spreads in the daily diet (Quilez et al., 2003; Winter, 2004). Spreads are also speculated not to be very convenient food vehicles for obtaining the optimal daily intake of phytosterols and stanols; the vehicle should be easier to incorporate into everyday life (Salo et al., 2002). Many new food types, including low-fat or even nonfat alternatives are therefore being investigated to the market of phytosterol/stanol-enriched foods. Examples of these products are presented in section 2.1.4. The use of different food vehicles with variable fat contents has, however, raised numerous questions concerning the cholesterol-lowering efficacy of free phytosterols and stanols, in particular, in these matrices. Some limitations in terms of efficacy have been reported, at least with nonfat liquid food vehicles.

A key factor in the function of phytosterols and stanols is the extent of their solubilization in the emulsified fat phase of food digest, i.e. they must be distributed throughout the fat phase to be effectively incorporated into the mixed micelles (Salo and Wester, 2005). When this process is analyzed from the viewpoint of fat-soluble phytosterol and stanol esters, solubilization is not a problem, instead the crucial step is hydrolysis of these esters by nonspecific pancreatic cholesterol esterase. The secretion of this enzyme is stimulated by dietary emulsion entering the intestinal

lumen, i.e. esters, especially in low-fat foods, should be consumed together with meals for better efficacy (Doornbos et al., 2006; Salo et al., 2005). In the case of free phytosterols and stanols, the solubilization step presents a problem. Thus, free sterols should be incorporated into fatty foods, unless they are activated by a suitable method like emulsification with lecithin (Ostlund, 2004). The efficiency of free phytosterols and stanols, as opposed to their esterified counterparts, seems to be more dependent on the food vehicle itself.

One of the first studies testing the suitability of low-fat aqueous food vehicles for phytosterol/stanol enrichment examined the administration of stanol esters (3 g/d of phytostanols) in low-fat (0.7%) yoghurt (Mensink et al., 2002). The results of this study showed that the LDL-cholesterol-lowering effect (nearly 14%) was comparable with that observed with stanol esters in oil-based products. This finding suggested that a high fat content in the food vehicle was not necessary for the efficacy of the phytostanol/sterol ester. Noteworthy is that these low-fat yoghurts were consumed with meals. Later, Doornbos et al. (2006) investigated the efficacy of phytosterol ester-enriched (2.8-3.2 g/d of phytosterols) single-dose yoghurt drinks with a fat content of 2.2% or 3.3% of total fat and with different intake occasions (with or without a meal). Their results indicated that the cholesterol-lowering effect of these products was independent of the fat content used, but the intake occasion was important; the reduction in LDL-cholesterol levels was 6.0% without a meal and 9.4% with a meal. Noakes et al. (2005) evaluated low-fat (0.54%) yoghurt enriched with phytosterol or stanol esters (1.7-1.8 g/d of phytosterols/stanols) and consumed as part of the normal diet and observed a 5-6% reduction in LDL-cholesterol. This reduction was considerably lower than that reported by Mensink et al. (2002) (see above). Noakes et al. (2005) concluded that even though there were differences between the phytosterol/stanol doses in these studies, the intake of phytosterols in conjunction with meals in the study of Mensink et al. (2002) could have enhanced efficacy of the phytosterols.

Low-fat milk has also been studied as a vehicle for free and esterified phytosterols. Thomsen et al. (2004) found that free phytosterol-enriched milk containing 1.2% milk fat and providing 1.2-1.6 g of phytosterols per day lowered serum LDL-cholesterol by 7-10% when consumed as part of the typical Danish diet. Clifton et al. (2004) investigated phytosterol esters (1.6 g/d of phytosterols) in milk containing 2% total fat and consumed across at least two meals and observed a 16% lowering in LDL-cholesterol levels. The reduction was greater than that observed for yoghurt- or cereal-based vehicles in the same study. The authors suggested that phytosterols probably are incorporated into the milk globule membrane, thus being easily available for transfer into the micellar membrane. In some other low-fat foods, phytosterols can be trapped in the centre of the lipid droplets and are unavailable until the fat is digested (Clifton et al., 2004).

The most controversial results concerning the LDL-lowering effect of phytosterols have been observed in nonfat liquid vehicles. Jones et al. (2003) reported that free phytosterols (1.8 g/d)

provided in low-fat (1%) or nonfat beverages and consumed with meals did not lead to higher reductions in total or LDL-cholesterol compared with a control diet. In a later study, however, the consumption of free phytosterol-enriched (2 g/d) nonfat orange juice consumed with breakfast and dinner meals led to a 12.4% reduction in LDL-cholesterol (Devaraj et al., 2004). The authors in the former study speculated that there should be more fat in the food vehicle to allow sufficient solubilization of phytosterols and better micellar absorption. Furthermore, they discussed that the use of solid food vehicles may be more effective since they allow more time for phytosterols to mix with gastrointestinal contents, which, in turn, provides better access to mixed micelles (Jones et al., 2003). Ostlund (2004) further analyzed the results of that study and concluded that the lack of efficacy of phytosterols was probably due to the absence of a proper formulation for free phytosterols.

2.1.4 Current spectrum of phytosterol-enriched foods

Since the launch of Benecol® margarine in Finland and Take control® in USA, the markets of phytosterol- and stanol-enriched foods have expanded enormously. For instance, Benecol® products are nowadays sold worldwide, and the product range varies from spreads to pasta and from milk-based drinks to cream cheese products (Salo et al., 2005). As noted in the previous section, the enriched food types are gradually changing from high-fat foods toward more healthy alternatives and attention has also been paid to making these foods easier to incorporate into the daily diet. Also under going development are food vehicles from which it is easy to estimate an adequate daily intake, e.g. single servings containing the optimal daily amount of phytosterols or stanols (Salo et al., 2002; 2005).

The overview given by web sites of such phytosterol manufacturers as Archer Daniels Midland Company (ADM) (www.admworld.com) (USA), Cargill Inc. (www.corowise.com) (USA), Cognis (www.cognis.com) (Germany), Forbes Medi-Tech Inc. (www.reducol.com) (Canada), Pharmaconsult Ltd. (www.multibene.com) (Finland), Raisio Plc. (www.benecol.net) (Finland), and Teriaka Ltd. (www.teriaka.com) (Finland) reveals that there are a large number of different phytosterol/stanol-enriched food types available around the world. Inside the EU, however, the regulatory process for phytosterol-enriched foods has slowed the introduction of new products and limited the number of food types available. As an example, of a total of 53 applications made between May 1997 and May 2004, only 14 novel foods were approved for commercialization (www.ec.europa.eu/food/food/biotechnology/novelfood). The regulatory process means that all novel foods or novel food ingredients planned to be placed on the market in the EU need to apply within the framework of Regulation (EC) No. 258/97 on novel foods and novel food ingredients (European Commission, 1997). Today, however, the approval process can also be simplified, only requiring notification from the company if their novel foods or novel food ingredients are

considered by a national food assessment body as substantially equivalent to existing foods or ingredients (www.ec.europa.eu/food/food/biotechnology/novelfood).

Since 'novel foods' are considered foods or food ingredients that have no significant history of consumption in the EU before 1997 (Palou et al., 2003), Benecol® products, which predate this EU regulation, have not been evaluated by the committee (SCF, 2003b). In USA, however, Benecol® has undergone an extensive evaluation in the late 1990s eventually receiving GRAS status in 1999 (Moreau et al., 2002). Food types enriched with the Benecol® ingredient as well as other phytosterol/stanol-enriched foods and food ingredients accepted for marketing in the EU are presented in Tables 1 and 2.

Table 1. Phytosterol/stanol ingredients approved by the European Commission for usage by member states up till spring 2006.

Brand names of ingredients/ manufacturer	Active components	Source of active components	Status[1]
Benecol®/ Raisio Plc.	Phytostanol esters	Tall oil, vegetable oils	GRAS 1999, in EU launched before regulation (EC) No. 258/97
CardioAid™/ Archer Daniels Midland (ADM)	Phytosterols, phytosterol esters	Soybean, rapeseed, palm, sunflower, and corn oils	GRAS 2001, EU Commission Decision, April 2004
Corowise™/ Cargill Inc.	Phytosterol esters, phytosterols, phytostanols	Vegetable oils	GRAS 2000, in EU accepted as substantially equivalent, December 2004
Danacol®/ Danone	Phytosterols, phytostanols	Information not available	In EU accepted as substantially equivalent, August 2004
Diminicol®/ Teriaka Ltd.	Phytosterols, phytostanols	Tall oil, vegetable oils	GRAS 2003, EU Commission Decision, April 2004
Multibene®/ Pharmaconsult Ltd.	Phytosterols/phytosterol esters and minerals	Tall oil, vegetable oils	EU Commission Decision, April 2004
Pro.activ™/ Unilever	Phytosterol esters	Soybean, corn, rapeseed, and sunflower oils	GRAS 1999, EU Commission Decision, August 2000
Prolocol/ Triple Crown	Phytosterols	Soybean oil, tall oil	In EU accepted as substantially equivalent, July 2004
Reducol™/ Forbes Medi-Tech Inc.	Phytosterols, phytostanols	Coniferous trees	GRAS 2000, EU Commission Decision, November 2004
Vegapure®/ Cognis	Phytosterols, phytosterol esters	Soybean, rapeseed, sunflower, and corn oils	In EU accepted as "substantially equivalent", August 2004

[1]GRAS status presented if information was available at www.cfsan.fda.gov. Information concerning acceptance in the EU has been gathered from the web site www.ec.europa.eu/food/food/biotechnology/novelfood.

Table 2. Phytosterol/stanol-enriched novel foods available/waiting for approval on the market in the EU in spring 2006.

Food type	Status	Reference[1]
Pasta[2]		www.benecol.net
Yellow fat spreads	EU Commission Decision, July 2000	European Commission, 2000
Milk-based fruit drinks	EU Commission Decision, March 2004	European Commission, 2004d
Milk-type products	EU Commission Decision, March 2004	European Commission, 2004a,b,c
Fermented milk-type products	EU Commission Decision, March 2004	European Commission, 2004a
Cheese-type products	EU Commission Decision, March 2004	European Commission, 2004a,d
Yoghurt-type products	EU Commission Decision, March 2004	European Commission, 2004b,c,d
Spicy sauces (including ketchup, mustard, marinades, dips, etc.)	EU Commission Decision, March 2004	European Commission, 2004b
Salad dressings (including mayonnaise)	EU Commission Decision, March 2004	European Commission, 2004a
Soya drinks	EU Commission Decision, March 2004	European Commission, 2004a
Milk-based beverages	EU Commission Decision, November 2004	European Commission, 2004e
Bakery products (rye bread)	EU Commission Decision, January 2006	European Commission, 2006a,b
Frankfurters, sausage, cold cuts	Waiting for approval since March 2000	www.ec.europa.eu/food/food/biotechnology/novelfood
Juices, nectars	Waiting for approval since October 2004	www.ec.europa.eu/food/food/biotechnology/novelfood
Rice drinks	Waiting for approval since October 2004	www.ec.europa.eu/food/food/biotechnology/novelfood

[1] Refers to the first European Commission decision concerning that food type.
[2] Pasta is enriched with Benecol® ingredient, i.e. European Commission approval was not needed

Phytosterol/stanol-enriched products that are or could be marketed outside the EU include juices, rice beverages, ice creams, snack bars, white or whole-grain breads and buns, cereals, confectionery products and cooking oils (information gathered from the web sites introduced above). In addition, GRAS status was recently given for phytosterol esters for use as an ingredient in ground roasted coffee (FDA, 2005), and for phytosterols and phytosterol esters for use in pasta, noodles, soups, and puddings (FDA, 2006a), and phytosterols for use in different egg products (FDA, 2006b).

This year, after a five-year regulatory process, one bakery product (rye bread) was approved for enrichment in the EU (European Commission, 2006a,b). Phytosterol-enriched juices and rice drink are still awaiting approval (Table 2).

The major growth area seems to be enrichment of dairy products, such as yogurts and milk drinks, although the first dairy food application, yoghurt enriched with phytostanol esters, appeared on the market in the United Kingdom already in 1999 (Salo et al., 2002). Low-fat dairy products are a convenient way for consumers to obtain the daily recommended amount of phytosterols/stanols because these products can be regarded as healthy staple foods, especially when milk fat is replaced with fat from phytosterol/stanol esters. Another benefit is the possibility to enrich single servings (Salo et al., 2002).

A few words about the future

Many foods can potentially be used as vehicles for phytosterols and stanols. An interesting enriched food type, especially from the viewpoint of this thesis, is cooking oils. The first phytosterol ester-enriched cooking oil "CookSmart™" was test-marketed in 2000 and 2001 (Moreau et al., 2002). At least one enriched cooking oil under the brand name Nextra Gold™ is currently being marketed in US to restaurants and food service companies (Watkins, 2005). Two other oils will probably come on the market: Reducol™-containing Vivola™ oil (www.forbesmedi.com) and phytosterol-enriched diacylglycerol oil under the brand name Enova™ (Moreau, 2004). The latter oil is already being sold under the Econa® brand name in Japan, as mentioned earlier. Described in the report of Winter (2005), a future research area may be the combination of phytosterols or stanols and some other beneficial ingredients such as soluble fiber.

2.1.5 Safety aspects

Phytosterols have been used for lowering plasma cholesterol levels for half a century now, and so far, no marked adverse effects have been reported (Ling and Jones, 1995; Katan et al., 2003; Berger et al., 2004; Kritchevsky, 2004; Gylling and Miettinen, 2005; Kritchevsky and Chen, 2005; Plat and Mensink, 2005; Salo et al. 2005). FDA has granted GRAS status for many phytosterol/stanol ingredients (FDA, 2000a,b,c; 2001; 2003; 2005; 2006a,b), and SCF in the EU has thoroughly reviewed the safety of phytosterols, phytostanols, and their esters and sanctioned them for use in a variety of foods (SCF, 2000; 2003a,b,c). Noteworthy, however, is that there is no experience of the effects of long-term consumption of phytosterols and stanols as part of the daily diet. This leaves open the possibility of unsuspected effects (Katan et al., 2003; St-Onge and Jones, 2003).

Safety evaluation is important within the EU: in order to ensure the highest level of protection of human health, novel foods must undergo a safety assessment before being placed on the EU market. Only those products considered to be safe for human consumption are authorised for marketing (www.ec.europa.eu/food/food/biotechnology/novelfood). Safety aspects include, for instance, the phytosterol profiles of the ingredients, which should follow the guidelines set by the Committee (SCF, 2003b). The ultimate end point of safety studies is defining the upper limit of consumption of

these compounds (Ratnayake and Vavasour, 2004). Research has been done on the absorption and toxicity of phytosterols and stanols and on the effect of these compounds on serum fat-soluble vitamin concentrations. Also the possibility of excessive intake of phytosterols and stanols has been estimated (Katan et al., 2003; Berger et al., 2004; Kritchevsky, 2004; Kritchevsky and Chen, 2005; Plat and Mensink, 2005).

Hepburn and coworkers have published an extensive series of safety evaluation studies of phytosterols and phytosterol esters as novel food ingredients (Baker et al., 1999; Waalkens-Berendsen et al., 1999; Weststrate et al., 1999; Ayesh et al., 1999; Hepburn et al., 1999; Wolfreys and Hepburn, 2002). The results of these studies are used to provide the backbone of safety information here (see below). To date, more data are available on phytosterol esters than on free phytosterols. However, since the physiologically active forms most likely are the free phytosterols, the safety data can be considered relevant for them as well (SCF, 2003b; Fahy et al., 2004). For instance, the results in a study of Delaney et al. (2004) indicated similar safety profiles of emulsified free phytosterols and phytosterol esters.

In the first part of the above-mentioned study series, Baker et al. (1999) examined the estrogenic potential of phytosterols and phytosterol esters using a combination of *in vitro* and *in vivo* assays. They showed that phytosterols do not bind to the estrogen receptor and observed no estrogenic activity *in vitro* when using the recombinant yeast strain or *in vivo* when the material was administered to immature female rats at doses of up to 500 mg/kg body weight/day. Nor were any unusual findings made when phytosterol esters were tested in a two-generation reproduction study in male and female rats at doses of up to 8.1% (w/w) of phytosterol esters (Waalkens-Berendsen et al., 1999). Weststrate et al. (1999), in turn, investigated the concentration of human fecal bile acids and sterols after subjects consumed a large amount of vegetable oil phytosterols (8.6 g phytosterols/d). This large intake increased the amount of phytosterols as well as cholesterol in the feces but reduced the concentration of bile acids. Taken together, the authors concluded that phytosterols may even have a beneficial effect on the risk of colon cancer. The same phytosterol consumption had no effect on either on human gut microflora or serum sex hormone levels in females (Ayesh et al., 1999).

Studies on subchronic toxicity have shown that phytosterol esters do not produce any general organ or systemic toxicity in Wistar rats at a dose of 6600 mg/kg body weight/day or less (Hepburn et al., 1999). However, at a higher level, 9000 mg/kg/day, the use of phytosterol esters resulted in the suppression of body weight gains in both sexes and in cardiomyopathy in male Sprague-Dawley rats (Kim et al., 2002). The authors of the former study concluded that 6600 mg/kg/day should be considered the no-observed-adverse-effect level (NOAEL) for phytosterol esters. The latter study led to the conclusion that the absolute toxic dose may be 9000 mg/kg/day. Noteworthy is that the relevance of the latter study to the human diet has been questioned since the health status of

experimental animals was compromised and the high amount of phytosterol esters tested was given as a bolus dose (SCF, 2003b).
The mutagenic and cytotoxic potential of phytosterols and phytosterols esters has also been assessed. No evidence of mutagenic activity was observed in internationally accepted *in vitro* assays or in two *in vivo* studies using rats (Wolfreys and Hepburn, 2002). Neither were any cytotoxic effects on Caco-2 cells seen when using membrane integrity assays (Fahy et al., 2004).

As summarized by Kritchevsky (2004) and SCF (2002), the results of safety studies with phytostanyl esters reflect those seen with phytosteryl esters. Phytostanyl esters have not, for instance, been shown to be genotoxic, and they have no estrogenic activity. Furthermore, no adverse effects on reproduction or development have been observed.

Absorption of phytosterols

The safety of phytosterols and stanols is to a large extent related to their lack of absorption from the intestinal tract. Minor absorption does, however, occur, but for healthy humans it usually remains considerably lower than that of dietary cholesterol, despite relatively similar natural dietary intakes (Thompson and Grundy, 2005). While absorption of cholesterol may vary from 30% to 80% (Bosner et al., 1999), the respective values for sitosterol, campesterol, stigmasterol, and sitostanol are around 5%, 15%, 5%, and 1% (Sanders et al., 2000). A double bond at C5-6 enhances absorption, whereas increasing the length of the side-chain group at C-24 diminishes absorption (Ostlund et al., 2002). The overall lower absorption of phytosterols and stanols may be due to their lower rate of esterification in enterocytes, limiting their incorporation into chylomicrons – compounds that mediate transport from enterocytes into the lymph (Trautwein et al., 2003).

The use of phytosterol-enriched foods leads to increased, but still low, serum phytosterol levels (Weststrate et al., 1998; Jones et al., 2000; De Graaf et al., 2002; Katan et al., 2003; Gylling and Miettinen, 2005; Kritchevksy and Chen, 2005). Absorption does not increase linearly with the increasing phytosterol dose, indicating that the absorption could be a saturable process (Ostlund et al., 2002). In addition, absorption of phytosterols may be separate from their effects on serum lipids. For instance, sterol-enriched bread elevated serum sterols as much as sterol-enriched milk, although the former had much smaller effects on serum LDL-cholesterol level (Clifton et al., 2004).

Currently, it is not known whether the circulating phytosterols or stanols have any physiologic or pathologic effects (Plat and Mensink, 2005). However, as the rare disease phytosterolemia is characterized by high serum phytosterol concentrations and increased risk for CHD, high dietary levels of phytosterols has been assumed to be atherogenic (Katan et al., 2003, Ratnayake and Vavasour, 2004; Plat and Mensink, 2005). This notion was supported by the finding that high plasma levels of phytosterols were associated with a positive family history of CHD (Sudhop et al.,

2002). However, controversial data have also been presented, i.e. no significant differences were measured between plasma sitosterol and campesterol levels for individuals with or without a family history of CHD (Wilund et al., 2004). More accurate information is anticipated with refinements in techniques for studying human atherosclerotic lesions (Kritchevsky and Chen, 2005).

Effect of phytosterols on serum levels of fat-soluble vitamins

Berger et al. (2004) summarized the results of 21 studies concerning the effects of free and esterified phytosterols as well as phytostanol esters on serum carotenoid status and fat-soluble vitamins. Some studies showed a significant reduction in the levels of carotenoids, tocopherol, and lycopene while others described no such effects. Katan et al. (2003) reported that in 18 trials (partly same as those of Berger et al., 2004) a significant reduction remained only for β-carotene (-12.1%), not for α-carotene (-0.3%) or lycopene (-0.1%), when the mean changes were adjusted for change in total serum cholesterol. More recently, in the study of Fahy et al. (2004), sitosterol supplementation had no effect on the content of α-tocopherol, but resulted in a significant decrease in β-carotene content when tested in Caco-2 cells. SCF (2000) reported that at phytosterol level of 11-13% in fat spreads no appreciable impact on the fat-soluble vitamins calciferol, tocopherols, or phylloquinone was apparent, although a 10% reduction occurred in α- and β-carotene and lycopene. However, a phytosterol level of 8% in spreads causes little or no drop in serum carotenoid levels.

As criticized by Kritchevsky (2004), consistency concerning data on changes in the above-mentioned fat-soluble nutrients is poor, although attempts have been made to standardize the data for all or some of the serum lipids. This standardization or correction should be made since carotenoids and tocopherols are known to be associated with LDL – and phytosterols and stanols reduce the amount of these LDL-particles (Moreau et al., 2002; Salo et al., 2005). Despite the lack of consistency, the reduction observed in serum carotenoid levels has been considered to be negligible compared with, for instance, seasonal changes (70%) in these nutrients (SCF, 2000; Kritchevsky and Chen, 2005; Noakes et al., 2005).

The clinical importance attributable to reduced levels of carotenoids or tocopherols is unknown (Kritchevsky, 2004; Plat and Mensink, 2005). Furthermore, no data exist on optimal levels of, for instance, carotenoids (Kritchevsky, 2005). Thus, the findings discussed above are rather difficult to evaluate. SCF (2000) has, however, reported that when vitamin A requirements are greater than normal, as for pregnant and lactating women and younger children, the concern with β-carotene level is probably more relevant.

The carotenoid-lowering effect of phytosterols and stanols has been speculated to be corrected by increasing the use of natural sources of β-carotene parallel with consumption of enriched foods (Berger et al., 2004). Indeed, in the study of Noakes et al. (2002), the increase in consumption of

high-carotenoid vegetables or fruits by ≥1 serving/d was sufficient to maintain serum carotenoid levels when phytosterol-enriched spreads was consumed. Miettinen and Gylling (2004), in turn, suggested that changes in vitamin absorption could be corrected by addition of fat-soluble vitamins to the phytostanol ester-enriched spread. Quilez et al. (2003) tested this kind of enrichment (α-tocopherol and β-carotene) with bakery products enriched with phytosterol esters and noted that the enrichment compensated for potential β-carotene deficiency.

Large intakes of phytosterols and stanols

The increasing selection of phytosterol and stanol-enriched food types has raised the question of whether some consumers might consume excessive amounts of these compounds. This can easily happen if each enriched food is designed to deliver the effective daily dose of phytosterols/stanols. The use of the recommended daily dose of, for instance, phytosterol-enriched yellow fat spread (20-30 g/d) already produces a phytosterol intake of about 1.6-2.4 g/d. This amount is 8- to 12- fold that derived from a natural daily intake of traditional products (SCF, 2000).

Evidence of problematic consumption has already emerged. Post-launch monitoring (Becel pro.activ™) presented by Unilever showed that while the use of enriched spread was lower than anticipated, only 15-18 g/d for regular consumers, some consumers were using both phytosterol- and stanol-enriched spreads. This indicates that situations in which several different phytosterol-enriched foods are used simultaneously may arise (SCF, 2002).

Attempts to estimate how high overall intakes can be have been made using simulation models. In the study of Raulio et al. (2001), the dietary intake data of 2862 Finns aged 25-64 years in 1997 were used. The intake of phytosterols/stanols was assessed by replacing 1-4 foods in the diet by their enriched counterparts. The results revealed that the daily intake of phytosterols can easily rise to over 4 g or even up to 9 g, especially in men. De Jong et al. (2004a) used in their simulation procedure a food frequency questionnaire distributed in 1993-1997 to 23106 Dutch people aged 20-60 years. They showed that when margarine, cheese, and yoghurt were replaced with the corresponding phytosterol/stanol-enriched products, the median daily intake of phytosterols/stanols was about 5.5 g in men and 4.6 g in women. However, in men, intakes of up to 9-10 g/d were also calculated.

A high intake of phytosterols could be a potential problem when these compounds are ingested together with cholesterol-lowering drugs. Although the combined use of phytosterol-enriched products and cholesterol-lowering drugs yields additional LDL-cholesterol reduction, long-term statin treatment has been shown to increase serum phytosterol concentrations. This is probably caused by reduced biliary secretion of phytosterols and increased absorption from the reduced intestinal cholesterol pool (Gylling and Miettinen, 2005). However, based on health behavior data

from Finland, the proportion of the population using both cholesterol-lowering drugs and phytosterol/stanol-enriched bread spreads was small and possible interactions were concluded not to be of major importance in terms of general public health risk (De Jong et al., 2004b).

2.2 Oxidative stability of phytosterols in foods

As a part of the above-mentioned safety assessments, the oxidative stability of phytosterols and stanols, i.e. their resistance to oxidation and thus to the formation of oxidation products, should be investigated. In terms of stability, the oxidative stability, indeed, is of major concern, since sterols, as unsaturated lipid compounds (steroid alcohols), are susceptible to oxidation (Labuza, 1971; Maerker, 1987; Porter et al., 1995; Dutta, 2004). Thus far, only a few studies have been performed on this area, although formation of oxidation products has been among the important safety questions for phytosterol-enriched products raised by EU member states (SCF, 2000). Furthermore, a report of Katan et al. (2003), summarizing the deliberations of experts on the efficacy and safety of sterols and stanols led to conclusion that if phytosterol/stanol-enriched products are to be marketed for frying purposes, their stability should first be established. Recently, in a review of genotoxicity of heat-processed foods, oxidized phytosterols were classified as cooked food mutagens, along with acrylamides, heterocyclic amines, nitrosoamines, and polyaromatic hydrocarbons (Jägerstad and Skog, 2005).

2.2.1 Formation of phytosterol oxidation products

Although cholesterol oxidation has been investigated for more than a century (Maerker, 1987), the first systematic studies on phytosterol oxidation were not conducted until the 1980s (Yanishlieva and Marinova, 1980; Yanishlieva-Maslarova et al., 1982; Yanishlieva and Schiller, 1983; Yanishlieva-Maslarova and Marinova-Tasheva, 1986). Thus, research on oxidation of phytosterols has not been as systematic and extensive as with cholesterol, but based on all information available, phytosterols seem to oxidize in a similar fashion to cholesterol. This can also be assumed from their structural similarities (Smith, 1987; Dutta and Savage, 2002a; Dutta, 2004). A foundation for understanding sterol oxidation mechanisms has been provided by studies conducted on fatty acid oxidation (Porter et al., 1995; Lercker and Rodriguez-Estrada, 2002).

Although sterols undergo oxidation, they are rather stable in pure state and also when heated alone at temperatures ≤100°C. This was shown by Osada et al. (1993) by heating cholesterol at 100°C; after 24 h of heating, virtually no sterol loss or formation of oxidized sterol was observed. At higher temperatures, however, sterol oxidation takes place easily, e.g. at 200°C after heating for 6 h, total degradation of sterol was reported (Osada et al., 1993).

In general, sterol oxidation can proceed via nonenzymic or enzymic pathways (Dutta, 2004). Nonenzymic pathways can further be divided into oxidation by a free radical mechanism, i.e. autoxidation, or oxidation by a nonradical mechanism, i.e. photoxidation (Lercker and Rodriguez-Estrada, 2002). In this section, only autoxidation, as a primary mechanism in oxidative deterioration of food lipids (Holman, 1954; Lundberg, 1962), is reviewed in detail.

Autoxidation of phytosterols

Mechanisms in sterol autoxidation are similar to those in fatty acid oxidation (Porter et al., 1995). The process is initiated by hydrogen atom transfer to generate carbon free radicals from the lipid substrate (Porter et al., 1995). This initiation reaction is not well understood, but active oxygen species, metals, and increased temperature are suggested to act as initiators (Labuza, 1971; Dutta et al., 1996). During the propagation step the free radicals formed react with molecular oxygen ($^3O_2$) to produce peroxyl radicals, and these, in turn, stabilize by hydrogen atom abstraction, yielding different sterol hydroperoxides. These primary oxidation products can then decompose to produce a variety of secondary oxidation products (Smith, 1987; Dutta et al., 1996).

The involvement of free radicals in sterol autoxidation has been confirmed with electron spin resonance investigation (Sevilla et al., 1986). Furthermore, lengthening of the induction period in the presence of antioxidants in aqueous cholesterol dispersions has indicated that sterol oxidation occurs via a free radical mechanism (Smith, 1987). The initiation of sterol autoxidation involves the formation of a carbon-centered C-7 radical due to the activating influence of a double bond between C-5 and C-6. From these radicals, the first detectable stable sterol oxidation products at room temperature, 7-hydroperoxides, form (Smith, 1987). Oxidative attack at another allylic position, C-4, seldom occurs due to a possible shielding effect provided by the neighboring hydroxyl group at C-3 and the trialkyl-substituted C-5 (Maerker, 1987).

During heating and/or storage sterol 7-hydroperoxides can decompose, yielding epimeric 7-hydroxysterols and 7-ketosterols (Smith, 1987; Lercker and Rodriguez-Estrada, 2002). The epimers of 5,6-epoxysterols are also important secondary oxidation products, but their formation occurs via a bimolecular reaction mechanism, the interaction of hydroperoxides and intact sterol (Smith, 1987; Lercker and Rodriguez-Estrada, 2002; Giuffrida, 2004). In many studies, 5β,6β-epoxysterol has prevailed over 5α,6α-epoxysterol, which is thought to be due to steric hindrance of the hydroxyl group at the C-3 position (Lercker and Rodriguez-Estrada, 2002). Although most sterol oxidation studies have been conducted on cholesterol, the secondary oxides presented here can be formed also during phytosterol oxidation (Yanishlieva and Marinova, 1981; Daly et al., 1983; Lampi et al., 2002). The systematic and trivial names of the major secondary oxidation products of the main phytosterols are presented in Table 3.

Table 3. Trivial and systematic names and commonly used abbreviations of the main secondary oxidation products of sitosterol, campesterol, and stigmasterol.

Trivial name	Abbreviation	Systematic name
Sitosterol		
7α-Hydroxysitosterol	7α-OH-sitosterol	(24*R*)-Ethylcholest-5-ene-3β,7α-diol
7β-Hydroxysitosterol	7β-OH-sitosterol	(24*R*)-Ethylcholest-5-ene-3β,7β-diol
5α,6α-Epoxysitosterol		(24*R*)-5α,6α-Epoxy-24-ethylcholestan-3β-ol
5β,6β-Epoxysitosterol		(24*R*)-5β,6β-Epoxy-24-ethylcholestan-3β-ol
7-Ketositosterol		(24*R*)-Ethylcholest-5-ene-3β-ol-7-one
Campesterol		
7α-Hydroxycampesterol	7α-OH-campesterol	(24*R*)-Methylcholest-5-ene-3β,7α-diol
7β-Hydroxycampesterol	7β-OH-campesterol	(24*R*)-Methylcholest-5-ene-3β,7β-diol
5α,6α-Epoxycampesterol		(24*R*)-5α,6α-Epoxy-24-methylcholestan-3β-ol
5β,6β-Epoxycampesterol		(24*R*)-5β,6β-Epoxy-24-methylcholestan-3β-ol
7-Ketocampesterol		(24*R*)-Methylcholest-5-ene-3β-ol-7-one
Stigmasterol		
7α-Hydroxystigmasterol	7α-OH-stigmasterol	(24*S*)-Ethylcholest-5,22-diene-3β,7α-diol
7β-Hydroxystigmasterol	7β-OH-stigmasterol	(24*S*)-Ethylcholest-5,22-diene-3β,7β-diol
5α,6α-Epoxystigmasterol		(24*S*)-5α,6α-Epoxy-24-ethylcholest-22-en-3β-ol
5β,6β-Epoxystigmasterol		(24*S*)-5β,6β-Epoxy-24-ethylcholest-22-en-3β-ol
7-Ketostigmasterol		(24*S*)-Ethylcholest-5,22-diene-3β-ol-7-one

Both epimers of 5,6-epoxysterols can further be converted to 3β,5α,6β-triol through hydration in an acidic environment (Maerker, 1987; Smith, 1987). Interestingly, the hydration of either epimer of these epoxides results in the formation of the same triol. However, the steric hindrance of C-5 by methyl at C-19 probably leads to a slower rate of hydration of 5α,6α-epoxysterol (Maerker, 1987). Other oxidation products of the sterol ring structure found in cholesterol oxidation studies but also in phytosterol oxidation studies include 6-hydroxy derivatives (e.g. (24*R*)-methylcholest-4-ene-3β,6β-diol), 6-keto derivatives (e.g. (24*R*)-methylcholestan-3β-ol-6-one), and epimeric 6-hydroxy-Δ^4-3-ketones (e.g. (24*R*)-methylcholest-4-ene-6β-ol-3-one) (Smith, 1987; Grandgirard et al., 2004a; Johnsson and Dutta, 2005).

In addition to the above-mentioned oxides, the oxidative attack can also occur at the tertiaric carbons in the side chain (Maerker, 1987). In phytosterol oxidation studies, 24-hydroxysterols (e.g. (24*S*)-ethylcholest-5,22-dien-3β,24-diol) and 25-hydroxysterols (e.g. (24*S*)-ethylcholest-5,22-dien-3β,25-diol) have been identified (Johnsson and Dutta, 2003; Johnsson et al., 2003). In cholesterol oxidation studies, 20-hydroxysterols (cholest-5-en-3β,20-diol) and 26-hydroxysterols (cholest-5-en-3β,26-diol) have also been found (Smith, 1987). The oxidations of both brassicasterol and stigmasterol with a double bond between C-22 and C-23 in the side chain have also been reported to lead to formation of 5,6;22,23-diepoxysterols (Giuffrida et al., 2004).

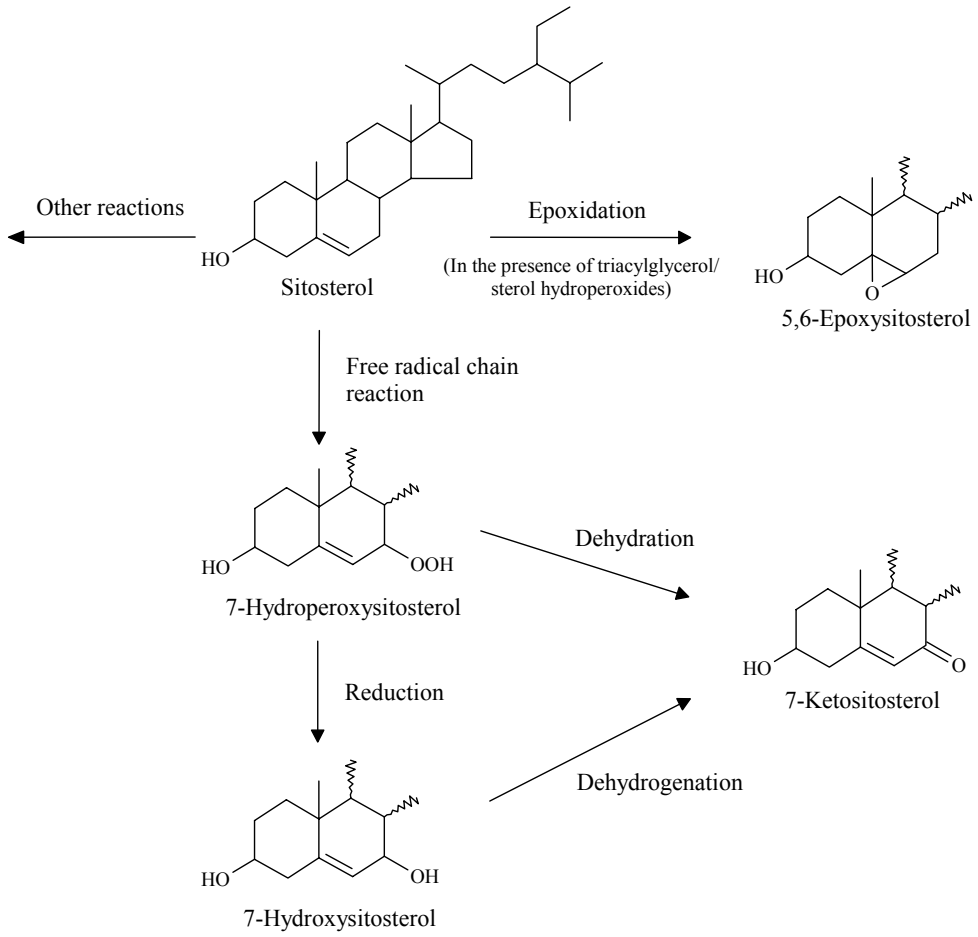

Figure 3. Reaction pathways for sitosterol oxidation. 7-hydroperoxy-, 7-hydroxy-, and 5,6-epoxysitosterols can undergo epimerization to form both α- and β-epimers.

Thus far, kinetic studies have only been conducted on allylic C-7 oxidation (Chien et al., 1998). In the cited study, in which cholesterol was oxidized by heating at 150°C for 30 min, the highest rate constant was observed for 7-hydroperoxysterol formation, followed by epoxidation, dehydration, reduction, and dehydrogenation (Figure 3).

Although the kinetics of lipid oxidation generally become more complex, for instance, in the presence of metals or at high temperatures (Frankel, 1998), the results of the above-mentioned kinetic study are in accordance with most cholesterol oxidation studies showing that 5,6-epoxysterols and 7-ketosterols are the most common oxides formed in either food or model systems (Tai et al., 2000).

Autoxidation of phytosterol esters

Autoxidation of sterol fatty acid esters involves the same free radical mechanism as that of oxidation of free sterols (Sevilla et al., 1986; Lercker and Rodriguez-Estrada, 2002). Furthermore, oxidation studies have revealed that sterol hydroperoxides mainly form in the allylic C-7 position,

also as in oxidation studies of free sterols (Lercker and Rodriguez-Estrada, 2002). In addition, the same major decomposition products, the epimers of 7-hydroxysterols, 5,6-epoxysterols, and 7-ketosterol, as well as triol, 20-hydroxysterol, 25-hydroxysterol, and 3,5-cholestadien-7-one derivatives, have been found to form during oxidation of cholesterol esters (Korahani et al., 1982). Different oxidation rates have, however, been observed. For instance, in aqueous alkali, free cholesterol oxidized faster than its esterified counterpart, but when heated in air or dissolved in oil, the situation was the opposite (Smith, 1987). Furthermore, in the solid state, the autoxidation rate of free cholesterol was much lower than that of cholesterol esters, but in water dispersion the oxidation rates were reversed (Korahani et al., 1987)

The autoxidation of cholesterol esters may result in the peroxidation of both the cholesterol moiety and the fatty acid moiety, meaning that three types of oxidized esters can be formed: an oxidized sterol moiety with an intact fatty acid moiety, an intact sterol moiety with an oxidized fatty acid moiety and an oxidized sterol moiety with an oxidized fatty acid moiety (Smith, 1996). Within the sterol ester molecule, a physical or a steric hindrance to oxidative attack has been postulated such that oxygen proceeds to the fatty acid moiety first, slowing the attack on the sterol moiety (Oehrl et al., 2001). Furthermore, oxidation of esters may proceed via an intramolecular oxidation system in which the first oxidized fatty acid moiety attacks the sterol moiety within the same molecule (Paniangvait et al., 1995).

Kinetic studies have shown that the initial rate of cholesterol ester oxidation increases with increasing unsaturation of the fatty acid moiety. However, the oxidation rate can decrease with time in such a way that after 10 h of heating cholesterol oxidizes more in the presence of a saturated fatty acid moiety (Korahani et al., 1982).

Although in the studies cited above only cholesterol esters have been investigated, phytosterol esters are known to oxidize as well. Research in this field has been done by Yanislieva and Maslarova (1980). They examined the kinetics of autoxidation of free sitosterol and sitosterol stearate in a pure state and found that at the beginning of the oxidation process, the ester form oxidized at a considerably higher rate than free sitosterol. However, during the subsequent stage of oxidation, when 7-hydroxysterols and 7-ketosterol accumulated in addition to peroxides, the rate of sitosterol oxidation was much higher. At 100°C, the autoxidation rates of sitosterol and sitosterol stearate were 1.1×10^{-3} and 2.1×10^{-2} meq/mole sec, respectively, during the initial stage of oxidation, while the corresponding maximum rates were 6.9×10^{-3} and 2.2×10^{-2} meq/mole sec. No other information is, however, available on phytosterol ester oxidation.

Autoxidation of phytostanols

Phytostanols as saturated compounds are considered to be less prone to oxidation than their unsaturated counterparts. Also, based on research on fatty acid oxidation, saturated compounds are known to often remain inert under mild oxidation conditions. However, at temperatures above 100°C, saturated lipids can undergo slow autoxidation. The oxidative attack is probably random and the primary products are hydroperoxides, which then decompose to yield, for instance, ketones and alcohols. Further oxidation and polymerization are also possible (Swern, 1961).

The oxidation mechanisms that have been suggested for phytostanols include side-chain oxidation and the generation of 3-keto compounds (Dutta, 2004). In addition, in their extensive work of dioxygenated C_{27}-, C_{28}-, and C_{29}-steroids, Aringer and Nordström (1981) have presented chromatographic and mass spectrometric properties of several saturated dihydroxy-C_{27}-steroids, 3-hydroxy-monooxy-C_{27}-steroids, and dioxo-C_{27}-steroids as well as some C-24 ethyl analogs of these oxides. So far, no other systematic research has been done in this field.

Formation of other decomposition products

In addition to the above-mentioned secondary sterol oxidation products, other decomposition products have been identified in sterol oxidation studies. These include sterol dehydration products (sterenes, i.e. sterol hydrocarbons), sterol dehydrogenation products (monooxygenated sterols with no hydroxyl group but with mono-, di-, or triunsaturation), and sterol condensation products like disteryl ethers (Lercker and Rodriguez-Estrada, 2002). Sterenes such as steradienes can form from intact sterol and have two double bonds at the 3,5, 2,4 or 2,5 positions. Steratrienes can form from sterol hydroxyl derivatives by the loss of two water molecules and have three double bonds located at the 2, 4 and 6 positions (Bortolomeazzi et al., 2000). Steratrienes with three double bonds at positions 3, 4, 22 have also been found (Dutta and Savage, 2002a). The dehydration of sterol 7-keto derivatives can lead to formation of steradiene with a keto group. Disteryl ethers, in turn, are produced by elimination of a water molecule from two sterol molecules (Lercker and Rodriguez-Estrada, 2002).

Decomposition products other than secondary oxides have also been observed to form in oxidation studies of cholesterol and phytosterols. This can be predicted from oxidation studies like the one conducted by Osada et al. (1993). The authors observed that when cholesterol was heated at 200°C, the formation of oxides remained rather low, but when heated at 150°C, the amount of oxides increased during the first 12 h of heating, decreasing thereafter. These observations suggest that cholesterol as well as cholesterol oxides were finally decomposed during heating.

The formation of other decomposition products was also observed by Kim and Nawar (1993). They oxidized pure cholesterol at 110-180°C for up to 80 h and found that the sum of major sterol oxidation products formed did not account for the cholesterol loss measured. Indeed, when they subjected a cholesterol sample, heated for 1 h at 180°C to size exclusion chromatography, they noted that a fraction comprising 21% of the initial cholesterol a represented material of higher molecular weight than the substrate. In their other study, a similar observation was made; total cholesterol oxides formed during heating at 180°C for 1 h accounted for only 30% of the cholesterol loss, i.e. the major portion of the cholesterol decomposition had occurred via some other pathway than the formation of secondary oxidation products (Nawar et al., 1991).

Yan and White (1990) heated cholesterol in a lard matrix and noticed that the total amount of cholesterol oxides formed was not equal to the amount of cholesterol loss. They suggested that many other decomposition products may be formed. Similar phenomenon was also observed in a phytosterol oxidation study (Oerhl et al., 2001). When vegetable oils were heated at 100-180°C for 20 h, the amount of phytosterol oxides detected after heating in different oils did not add up to the amount of phytosterols lost. The authors speculated that one reason could be polymer formation, although some analytical problems may also have affected the results (Oerhl et al., 2001). Considering the findings of cholesterol oxidation studies to date, more information is needed to resolve the stability of phytosterols during long-term heating.

2.2.2 Formation and content of phytosterol oxidation products in foods

As early as in the 1960s, evidence was gained of the presence of phytosterol oxidation products in foods: both phytosterol autoxidation products and sterol hydrocarbons were observed to form during refining of vegetable oils (Niewiadomski and Sawicki, 1964; 1966). In general, due to the free radical process of sterol autoxidation, any sterol-containing food exposed to oxidation-promoting factors, such as light, heat, air, water, and metal ions, should be suspected as a possible source of sterol oxidation products (Finocchiaro and Richardson, 1983; Maerker, 1987). These factors are commonly encountered during food processing and storage. Furthermore, enzymic oxidation of sterols prior to food processing and storage cannot be totally excluded (Dutta et al., 1996).

Due to the complexity of food as a matrix and sterol oxidation as a phenomenon, many challenges exist for studies of sterol oxidation in foods. Investigations conducted on pure sterols differ from those carried out on real foods since numerous factors, including the presence of other lipids, water, protein, and food components, can affect sterol oxidation (Chien et al., 1998). Furthermore, sterol oxidation is system-dependent, e.g. temperature, oxidation time, and pH can greatly affect the formation and distribution of sterol oxidation products (Kim and Nawar, 1993). To overcome these problems and to obtain a better understanding on the oxidative behavior of sterols in foods, sterol

oxidation has also been examined in simplified model systems. Generally, however, knowledge about phytosterol oxidation in foods is scarce, with comprehensive data in this field being lacking.

Phytosterol oxidation in food model systems

Blekas and Boskou (1989) heated stigmasterol (5%) in a triacylglycerol mixture at 180°C for several hours and identified the formation of eleven stigmasterol oxidation products, including 7-hydroxides and 5,6-epoxides, as well as sterol hydrocarbons such as stigmasta-3,5,22-triene, stigmasta-3,5,22-trien-7-one, and stigmasta-4,22-dien-3-one. Similar products were found to form from sitosterol (5%) heated in tristearin, lard, and sunflower oil at 120°C for 2 h. In the latter study, the percentage of sitosterol oxidation was also noted to increase with increasing unsaturation of the lipid matrix; however, the more saturated the matrix was, the more phytosterols actively participated in autoxidation compared with triacylglycerols (Yanishlieva-Maslarova and Marinova-Tasheva, 1986).

By measuring the loss of phytosterols, it has been clarified that the oxidative stability of phytosterols is determined by the saturation degree of the sterol ring structure, i.e. sitostanol with a saturated ring structure was the most stable and ergosterol with two double bonds in the ring structure the least stable when heated at a level of 0.1% in mineral or rapeseed oil at 180°C for 24 h. At this high temperature, the loss of all phytosterols investigated increased with increasing saturation of the lipid matrix (Lampi et al., 1999). Similar observations were made when phytosterols were heated in tripalmitin and RSO at 180°C; in tripalmitin <10% of phytosterols were found after 9 h of heating, while in RSO approximately 80% of sterols were left (Lampi et al., 2000).

Both the loss of phytosterols and the accumulation of phytosterol oxides were measured in canola, coconut, peanut, and soybean oils heated at 100°C, 150°C, and 180°C for 20 h. To monitor the formation of phytosterol oxides, oils were also enriched with an additional 90 mg/100 g of sitosterol before heating. The phytosterol content was observed to decrease with increasing temperature. For instance, in canola oil, heated at 100°C, the losses for sitosterol and campesterol were 32% and 33%, and at 150°C 94% and 95%, respectively. Furthermore, the losses were greater in canola and soybean oils than in the more saturated oils. For example, at 100°C, 56% of campesterol was lost in soybean oil, while only 8% of campesterol and 16% of sitosterol were lost in coconut oil. However, at 180°C, the losses in coconut oil were much greater with 98% of sitosterol and 97% of campesterol being lost (Oehrl et al., 2001).

In the same study, heat treatments were so drastic that phytosterol oxides also started to decompose, especially during heating at 150°C and 180°C. At 100°C, the main phytosterol oxides detected were the epimers of 5,6-epoxysitosterols and –campesterols, and their amounts were in the range of 1.1-

3.8 mg/100 g of enriched canola oil. After 20 h of heating at 150°C, no epoxyphytosterols were detected, but 1.5 mg/100 g of 7α-hydroxysitosterol and 0.3 mg/100 g of 7-ketocampesterol were present (Oehrl et al., 2001).

In the studies cited above, interesting interactions between phytosterols and lipid matrices were observed. These findings are supported by cholesterol oxidation studies. For instance, when mixtures of cholesterol and tristearin, triolein, trilinolein, or milk fat (1:1) were heated at 130°C for 3 h, the loss of cholesterol was the greatest in triolein, while tristearin was the least destructive, i.e. sterol loss increased with increasing unsaturation (Kim and Nawar, 1991). In contrast, another study showed that at higher temperature, 180°C, the sterol loss was greater in a cholesterol/tristearin (1:1) mixture than in a cholesterol/trilinolein mixture (Nawar et al., 1991). Interactions between sterols and triacylglycerols are speculated to be influenced by the relative ratios of the oxidizing components, the dispersion system, the oxidation conditions, and the oxidation status of the different components (Lercker and Rodriguez-Estrada, 2002).

Phytosterol oxidation in food products

Thus far, phytosterol oxidation products in foods and the effects of processing and storage on these products have been studied relatively little. Many reports in this field deal with the effect of oil refining on phytosterol contents in oils. In these studies, mainly phytosterol loss has been measured and the loss observed has, at least partly, been due to physical removal of sterols during refining, i.e. the loss through autoxidation does not seem to be so important (Piironen et al., 2000). Indeed, the analysis of freshly refined soybean oil showed no increase in phytosterol oxide levels compared with crude oil (Nourooz-Zadeh and Appelqvist, 1992).

In more recent studies, the formation of phytosterol oxides was investigated in low erucic acid RSO (Lambelet et al., 2003) and also in peanut, sunflower, corn, and lampante olive oils (Bortolomeazzi et al., 2003) during refining. In the former study, the total amount of oxidized phytosterols in semirefined low erucic acid RSO was 70 μg/g, and after deodorization 69-74 μg/g depending on the processing temperature. The oxides found were mainly oxidized at the C-7 position (Lambelet et al., 2003). In the latter study, the phytosterol oxide contents in crude peanut, sunflower, corn, and lampante olive oils fell within 2.6-9.6 μg/g, 4.5-67.5 μg/g, 4.1-60.1 μg/g, and 1.5-5.5 μg/g, respectively, consisting mainly of 7-hydroxysterols and 7-ketosterols. Bleaching caused a significant reduction in the levels of 7-hydroxysterols with partial formation of sterol hydrocarbons (steratrienes). After deodorization, however, no changes in phytosterol oxides and no formation of dehydration products were observed (Bortolomeazzi et al., 2003).

In some studies, the formation of phytosterol oxides in refined vegetable oils at cooking and frying temperatures as well as during long-term storage has also been investigated. The results of these

studies have been highly variable due to the use of many different oxidation methods. The surface-to-volume ratio, an important factor in terms of oxidation, may also vary considerably.

Lampi et al. (2002) measured sitosterol and campesterol oxidation in RSO heated at 180°C for up to 24 h. Oxidation of these phytosterols was observed to proceed slower than that of stigmasterol in a bulk state. The amounts of total and individual oxides increased steadily during the experiment. After 24 h of heating, the total amounts of sitosterol and campesterol oxides (7-hydroxysterols, 5,6-epoxysterols, 7-ketosterol, and 25-hydroxysterol) were 646 μg/g and 452 μg/g of oil, respectively. Interestingly, the ratio of these oxides followed the ratio of intact sitosterol and campesterol in unheated RSO. The main products formed were 7β-hydroxy and 5β,6β-epoxy derivatives (Lampi et al., 2002).

When sunflower and olive oils were heated at 150°C and 200°C for up to 60 min, the total amounts of sitosterol oxides (7-hydroxysterols, 5,6-epoxysterols, 7-ketosterol, and triol) were 241 μg/g and 815 μg/g in sunflower oil and 37 μg/g and 365 μg/g in olive oil, respectively. The major product throughout the heating in all samples was 7-ketositosterol (Zhang et al., 2005a). In another study, soybean, olive, corn, and peanut oils were heated at 180°C for various times, and the amounts of total phytosterol oxides in these oils after, for instance, 60 min of heating were 24, 32, 77, and 77 μg/g of oil (Dutta and Savage, 2002a).

Dutta (1997) examined phytosterol oxide contents in a RSO/palm oil blend, sunflower oil, and high-oleic sunflower oil before and two days after the usage of these oils in frying of French fries. 7-hydroxy, 5,6-epoxy, 7-keto, and triol derivatives of sitosterol were observed, as were traces of campesterol and stigmasterol oxides. The main oxides formed seemed to vary in different oils, being 5,6-epoxysterols, 7-ketosterol, or 7α-hydroxysterol. In all oils, the oxide content slightly increased during two days of storage, being 59, 57 and 56 μg/g in the RSO/palm oil blend, sunflower oil, and high-oleic sunflower oil, respectively (Dutta, 1997). For comparison, storage of freshly refined soybean oil at 4°C for one year caused no significant increase in sitosterol oxide levels as compared with the initial state, i.e. oxide levels remained below the detection limit of 0.2 μg/g (Nourooz-Zadeh and Appelqvist, 1992). However, storage of sunflower oil in a transparent bottle for 40 years led to similar phytosterol oxidation as that observed in the presence of air at 100°C after 13 h of heating. Furthermore, oxidative changes in phytosterols were considerably higher than in fatty acids (Yanishlieva and Schiller, 1983).

Other foods in which phytosterol oxidation has been investigated include phytosterol ester-enriched spreads, coffee, wheat flour, infant milk formulas, French fries, and potato chips. It is, however, noteworthy that although quantitative results have been presented, mostly these results are only rough estimates. In spreads, the total amount of phytosterol oxides observed has been reported to be 12 μg/g (Johnsson and Dutta, 2006), 68 μg/g (Grandgirard et al., 2004a), and 47 μg/g of spread

(Conchillo et al., 2005). The oxides identified in these samples include 7-hydroxysterols, 5,6-epoxysterols, 7-ketosterols, and triols (Grandgirard et al., 2004; Conchillo et al., 2005; Johnsson and Dutta, 2006); 24-hydroxysterols (Johnsson and Dutta, 2006), 6-hydroxysterols, and 6-ketosterols have, at least tentatively, also been identified (Grandgirard et al., 2004a).

Levels of sitosterol and campesterol oxides in potato chips fried in palm oil, sunflower oil, and high-oleic sunflower oil and stored for 0-25 weeks at room temperature in the dark have also been investigated. The initial levels of oxides in these chips were 5, 46, and 35 μg/g of lipids, respectively. In a storage experiment, the highest oxide levels were measured in chips fried in high-oleic sunflower oil and stored for 25 weeks: 59 μg/g of lipids (19 μg/g of chips) consisting of 7-hydroxy-, 5,6-epoxy-, 7-keto-, and triol derivatives of sitosterol and campesterol (Dutta and Appelqvist, 1997). Lee et al. (1985) evaluated the formation of phytosterol oxides in high-temperature storage (40°C) of potato chips fried in cottonseed oil. They found sitosterol oxides to appear after 95 days of storage, the major oxides being 7α-hydroxysitosterol (13 μg/g of lipids), 7β-hydroxysitosterol (9 μg/g of lipids), and 5β,6β-epoxysitosterol (6 μg/g of lipids). At a lower temperature, 23°C, even storage for 150 days failed to produce any detectable amounts of oxides. The detection limit was, however, not specified.

In French fries fried in a RSO/palm oil blend, sunflower oil, and high-oleic sunflower oil, the oxide contents (7-hydroxy-, 5,6-epoxy-, 7-keto-, and triol derivatives of sitosterol and campesterol) were 32, 37, and 54 μg/g of lipids, respectively, corresponding to 2.4, 2.8 and 4.0 μg/g of French fries (Dutta, 1997). In another study, French fries were fried in similar oils and the total amounts of oxides of sitosterol, campesterol, and stigmasterol formed in a RSO/palm oil blend, sunflower oil, and high-oleic sunflower oil were 191, 39, and 69 μg/g of lipids, respectively (Dutta and Appelqvist, 1996a). The authors concluded that differences observed between these studies were due to different final frying temperatures. In the former study, French fries were prepared for consumption by heating at 200°C and in the latter at 250°C for 15 min.

In coffee and infant milk formulas, only the amount of 7-ketositosterol was measured and its level remained below 0.04 μg/g of lipids in coffee (Turchetto et al., 1993) and 4 μg/g of lipids in milk formulas (Zunin et al., 1998). In wheat flour, the generation of sitosterol oxides (5,6-epoxysterols and 7-hydroxysterols) during 2, 8, and 36 months of storage at room temperature was analyzed. The total amounts of oxides measured after these storage experiments were 35, 24, and 328 μg/g of lipids, respectively. The samples were, however, from different batches, thus, the oxide contents were not fully comparable (Nourooz-Zadeh and Appelqvist, 1992).

2.2.3 Biological effects of phytosterol oxidation products

Extensive research has been conducted on the biological effects of cholesterol oxidation products. Evidence exists that these oxides may be linked to a series of human diseases by exerting a deleterious effect on lipid metabolism and cell function (Osada, 2002). Hundreds of *in vitro* studies have shown activities of cholesterol oxides at the onset and during progression of arteriosclerosis (Garcia-Cruset et al., 2002), although research in this field is too limited to allow any definite conclusions to be drawn (Schroepfer, 2000; Björkhem and Diczfalusy, 2002; Olkkonen and Lehto, 2005). Cholesterol oxides can also show cytotoxic, mutagenic, and carcinogenic activities (Osada, 2002).

Due to structural similarities, phytosterol oxides are thought to possess harmful features analogous to those of cholesterol oxides. Compared with the amount of research completed on cholesterol oxides, phytosterol oxides have, however, received scant attention and no data are available on phytostanol oxides. This field of study is now beginning to expand. In terms of the oxidative stability of phytosterols in foods, the lack of information on how oxidized sterols end up in the human body makes these stability studies even more important. Research on cholesterol oxides has indicated that oxides are mainly of dietary origin, but also partly formed *in vivo* (Osada, 2002). A recent study revealed that in Wistar rats at least sitostanetriol and campestanetriol were not formed *in vivo* although they were present in serum (Grandgirard et al., 2004b). With increasing consumption of phytosterol-enriched foods, more information is thus needed on the origin of serum phytosterol oxides, their health effects, and the prevention of their formation in foods.

To date, most studies on the biological effects of phytosterol oxides have concentrated on toxicity of these compounds. Meyer et al. (1998) tested the cytotoxicity of phytosterol and cholesterol oxides by measuring the mortality of mealworms. The activities of the sitosterol and stigmasterol oxides evaluated were rather similar, but were five times lower than those of cholesterol oxides. Furthermore, the epimers of 5,6-epoxysitosterol and 5,6-epoxystigmasterol showed equal activities, but were only half of that of the corresponding triols, which developed a 40% mortality in 5×10^{-3} M solution after 23 h. The cytotoxicity study conducted in cultured macrophage-derived cell lines indicated similar patterns of toxicity for all major cholesterol, sitosterol, and campesterol oxides. In addition, by comparing cell viability and lactate dehydrogenase leakage, cells were observed to recover more readily from the administration of intact cholesterol and phytosterols than their oxides (Adcox et al., 2001).

Maguire et al. (2003) studied cytotoxicity, apoptosis, antioxidant status, and genotoxicity of sitosterol oxides on a human monocytic blood cell line (U937 cells). Depending on the concentration, sitosterol oxides as a mixture demonstrated biological effects similar to 7β-hydroxycholesterol towards U937 cells, i.e. reduced cell viability, induced apoptosis, and decreased

glutathione levels. No genotoxic effects were, however, observed. Interestingly, 5α,6α-epoxysitosterol tested as a single compound showed no toxicity towards U937 cells. The authors concluded that when evaluating toxicity it should be borne in mind that single oxides may behave differently from oxide mixtures. They also emphasized that the hierarchy of the toxicity of oxides needs to be established.

In a more recent study, individual phytosterol oxidation products were, indeed, synthesized and their cytotoxicity and apoptotic potential were tested on U937, CaCo-2, and HepG2 (human hepatoma) cell lines (Ryan et al., 2005). Among the tested oxides (5α,6α-epoxysitosterol, 5β,6β-epoxysitosterol, 7β-hydroxysitosterol, 7-ketositosterol, and sitostanetriol), 7-ketositosterol and 7β-hydroxysitosterol were found to be the most cytotoxic, while 5α,6α-epoxysitosterol showed no toxicity at all. Generally, toxic effects were similar to those of cholesterol oxides, but higher amounts of phytosterol oxides were required for comparable levels. In addition, it was observed that apoptotic pathways provoked by cholesterol and phytosterol oxides can be different (Ryan et al., 2005).

The genotoxicity and subchronic toxicity of phytosterol oxides have been assessed in *in vitro* assays using *Salmonella typhimurium* strains and human lymphocytes, and in a 90-day feeding study in rats. The phytosterol oxide concentrate used in this study (30% oxides) did not possess genotoxic potential in *in vitro* assays and showed no evidence of subchronic toxicity when administered to Wistar rats at a mean dietary phytosterol oxide concentration of 0.44-0.45% (w/w). These findings led to a NOEL (no-observed-effect level) at an estimated dietary level of phytosterol oxides of 128 mg/kg/day for males and 144 mg/kg/day for females (Lea et al., 2004).

Absorption

Studies on phytosterol oxide absorption are also scarce. Due to the low intestinal absorption of phytosterols, the action of their oxidation products has not been of particular interest. But, again, enrichment of foods and the possible formation of phytosterol oxides in these products have changed the situation on this front. The new focus is to resolve how these oxides are metabolized and excreted and whether they are incorporated into tissues (Grandgirard, 2002).

Grandgirard et al. (1999) investigated the lymphatic absorption of 7-ketositosterol and campesterol and the epimers of 5,6-epoxysitosterol and campesterol in male Wistar rats. 7-keto derivatives were observed in lymph in very low but similar quantities to those of intact phytosterols. The mean absorption value was 1.5% of the ingested phytosterol oxides; however, a large variation was observed. Interestingly, 7-ketocampesterol was more efficiently absorbed than 7-ketositosterol. The increasing length of the side-chain group at C-24 thus diminished oxide absorption, as also observed for intact phytosterols (Ostlund et al., 2002). All 5,6-epoxyphytosterols as well as one new

compound, stigmastentriol, were also present in the lymph. The authors hypothesized that 5,6-epoxysterols had converted to triols inside the intestinal cells. Compared with 7-keto derivatives, 5,6-epoxysterols were absorbed better (4.7%). Furthermore, 5β,6β-epoxyphytosterols were absorbed more efficiently than their 5α,6α-epimers, and campesterol derivatives better than sitosterol derivatives (Grandgirard et al., 1999).

More recently, lymphatic absorption of 7-hydroxy, 5,6-epoxy, 7-keto, and triol derivatives of sitosterol and campesterol were evaluated in Sprague-Dawley rats. Also in this study lymphatic recoveries of campesterol oxides were higher than those of sitosterol oxides, but all phytosterol oxides were absorbed less than the corresponding cholesterol oxides (Tomoyori et al., 2004). The highest recovery in the case of phytosterol oxides was observed for 7-hydroxy derivatives. Furthermore, the recovery of phytosterol oxides was found to be higher than that of intact phytosterols. In the same study, also the effect of phytosterol oxides on atherosclerosis in apolipoprotein E-deficient mice was examined. Although diet-derived phytosterol oxides accumulated in the serum, liver, and aorta of mice, they did not promote the development of atherosclerosis (Tomoyori et al., 2004).

More information on accumulation of phytosterol oxides in tissues from the diet was obtained from the study of Grandgirard et al. (2004c). They fed hamsters with mixtures of sitosterol or campesterol oxides (mainly 7-hydroxy, 5,6-epoxy, 7-keto derivatives) and recovered oxides from the plasma, aorta, liver, kidneys, and heart. Levels found were noticeable when hamsters were fed 2500 μg/g oxides in their diet, but with a diet containing 100 μg/g oxides only sitostanetriol was recovered. 5α,6α-epoxysterols and 7α-hydroxysterols were observed in no tissues other than plasma. The authors suggested that these derivatives were probably better metabolized *in vivo* than the other oxides. It is also possible that 5α,6α-epoxysterols are transformed in triols (Grandgirard et al., 2004c).

Interestingly, oxidized phytosterols have been found in the serum of a patient with phytosterolemia (Plat et al., 2001) as well as in the serum of healthy humans (Grandgirard et al., 2004d). In the former, study 7-ketositosterol, the epimers of 5,6-epoxysitosterol, 7β-hydroxysitosterol and sitostanetriol were present in the range of 0.08-2.77 μg/ml of serum. Also 7α-hydroxysitosterol and the epimers of 7-hydroxycampesterol were found, but due to analytical problems these findings were not entirely reliable. In the latter study, the epimers of 5,6-epoxysitosterol, campestanetriol, sitostanetriol, and 7-ketositosterol were found in the serum of healthy humans, although their amounts were significantly lower than those observed for phytosterolemic patient; the mean values varied from 4.8 ng/ml of serum for campestanetriol to 57.2 ng/ml of serum for 5β,6β-epoxysitosterol. An important note is that in both studies the oxides found from serum were mainly of sitosterol origin.

2.3 Analysis of phytosterol oxidation products in foods

No validated, internationally recognized methodology exists for the analysis of phytosterol oxidation products in foods. The analytical methods used are based on those developed for cholesterol oxidation products (Piironen et al., 2000; Dutta, 2002; Zhang et al., 2005a), although no validated methods are available for these compounds either (Appelqvist, 2004).

Compared with cholesterol oxides, the development of accurate methods for qualitative and quantitative analyses of phytosterol oxides in foods is even more challenging. Higher selectivity is needed since the number of different oxides formed from phytosterols is at least 3-4 times higher than those derived from cholesterol. This is because phytosterols are present as a mixture both in natural and enriched foods, and all phytosterols in these mixtures can oxidize (Dutta, 2002). Higher sensitivity is also needed since many of these phytosterols are present in low quantities, and thus, the amount of their oxidation products can also be low.

To date, gas chromatographic (GC) methods have been the most frequently employed techniques for the analysis of sterol oxides in foods (Dutta et al., 1996; Guardiola et al., 2004). In addition, as a result of an interlaboratory study on cholesterol oxides in foods, a recommendation was recently made of using GC both in qualitative and quantitative analysis of sterol oxides (Appelqvist, 2004). For these reasons, only the applications of GC methods on phytosterol oxide analytics are presented in the next sections.

2.3.1 Sample preparation

Since phytosterol oxides occur in foods mainly at μg/g levels, extensive pre-treatments are needed for their accurate determination with chromatographic methods. As in cholesterol oxidation studies, a multi-step sample preparation includes 1) extraction of lipids from food materials, 2) saponification of lipids, and 3) purification and enrichment of phytosterol oxides (Piironen et al., 2000; Guardiola et al., 2002; Dutta, 2004). A final step before chromatographic separation, detection, and quantification is derivatization of the samples (Guardiola et al., 2002). Generally, throughout the sample preparation procedure, care must be taken to minimize further reactions of phytosterol oxides; i.e. high temperatures, light, and oxygen contact should be avoided (Piironen et al., 2000).

Extraction of lipids

The isolation of phytosterol oxides from food matrices usually starts with extraction of lipids if samples are not already in the form of lipid extracts – like vegetable oils. To obtain good recoveries for sterol oxidation products, careful extraction is crucial. Attention should be paid to modifying the

extraction procedures to suit for different food matrix compositions and different polarities of oxides ranging from almost nonpolar 7-ketosterols to polar 3β,5α,6β-triols (Dutta et al., 1996). Usually, extraction of the lipid fraction containing phytosterol oxides has been performed with hexane/isopropanol, but other solvents or solvent mixtures have also been used (Table 4). When Dutta and Appelqvist (1996a) compared different solvents in lipid extraction from potato crisps, they found no marked differences between hexane (100%), chloroform/methanol (2:1, v/v), and hexane/isopropanol (3:2, v/v). They chose to use the hexane/isopropanol mixture, probably because it is a less toxic option.

It is also possible to omit lipid extraction and start the isolation procedure with direct saponificaton or transesterification of the food sample. Interestingly, when the direct method was compared with chloroform/methanol (2:1, v/v), hexane/isopropanol (3:2, v/v), and dichloromethane/methanol (9:1, v/v) extractions (column chromatography), the procedure offering the best compromise, at least in cholesterol oxide analysis in milk powders, was direct saponification, which had minimal artifact formation and good repeatability and accuracy. This method also involved less solvents and a shorter analysis time (Dionisi et al., 1998).

Saponification of lipids

In lipid extracts, the predominating compounds are triacylglycerols, but phospholipids and unoxidized sterols, for instance, can also be present. In order to separate phytosterol oxides from acylglycerols, saponification is needed. The procedure is carried out in an alkaline medium, and, as a result, acylglycerols convert into water-soluble compounds, and sterol esters, if present, hydrolyze. When direct saponification is applied, the alkaline medium is added directly into the food sample (Dionisi et al., 1998). The remaining unsaponifiable matter, consisting of free sterols and sterol oxides, can be recovered by extraction with a suitable solvent (Dutta et al., 1996; Ulberth and Buchgraber, 2002). As presented in Table 4, the most commonly used solvents in phytosterol oxide analytics are dichloromethane and diethyl ether. The solvent selected should be somewhat polar since the common sterol oxides are more polar than native sterols.

To facilitate the hydrolysis of the lipid matrix, the reaction mixture could be heated. Hot saponification can, however, cause the artifactual formation of 7-hydroxysterols and 7-ketosterol or the dehydration of 7-ketosterol to form steradiene with a keto group (Guardiola et al., 2004). Saponifying at room temperature, i.e. cold saponification, is thus recommended, although it is time-consuming. As can be seen in Table 4, cold saponification using ethanolic or methanolic potassium hydroxide (KOH) is widely used when analyzing phytosterol oxides in foods. No artifact formation has been observed during cold saponification (Louter, 2004), but in terms of destruction of 7-ketosterol, controversial data exist. Some studies have described extensive losses, while others have reported negligible changes. To clarify this conflicting information, a kinetic evaluation of 7-

ketocholesterol stability during saponification was carried out by Park et al. (1996). The authors reported that even short heating might significantly lower the recovery of the 7-ketocholesterol, but saponification in 1N methanolic KOH medium at RT for 18 h yields to virtually negligible losses of the analyte (Park et al., 1996).

One new approach for the isolation of phytosterol oxides is transesterification. This procedure involves the cleavage of ester bonds of acylglycerols and sterol esters, followed by the formation of methyl esters. When transesterification was applied in a phytosterol autoxidation study (Johnsson and Dutta, 2006), no artifact formation was observed with thin-layer chromatography (TLC), but for sufficient separation of phytosterol oxides from fatty acid methyl esters a two-fold solid-phase extraction (SPE) was needed. The authors concluded that this method was rapid, efficient, and fully comparable with other existing methods in terms of recoveries of both medium polar and polar phytosterol oxides (Johnsson and Dutta, 2006).

Purification and enrichment of phytosterol oxides

To further reduce the amount of material in the final chromatographic separation, chromatographic sample clean-up is commonly used in conjunction with saponification in determination of sterol oxides (Guardiola et al., 2004). Particularly, isolation of phytosterol oxides of any of the unoxidized phytosterols is important to avoid coelution in GC (Dutta and Appelqvist, 1997). Nowadays, chromatographic clean-up is mainly carried out in disposable ready-to-use SPE cartridges with different phases (Ulberth and Buchgraber, 2002; Guardiola et al., 2004). For sterol oxides, silica and aminopropyl phases have shown the best efficiency and reproducibility (Piironen et al., 2000). Since SPE exploits differences in the polarity of interfering compounds and analytes, adequate separation is achieved by stepwise elution with increasing solvent polarity (Piironen et al., 2000; Guardiola et al., 2004).

Phytosterol oxides are more polar than the corresponding sterols. Noteworthy, however, is that sterols are only slightly less retained with respect to 25-hydroxysterols, 7-ketosterols, and 5,6-epoxysterols, while triol derivatives are strongly retained. To achieve complete separation between oxides and unoxidized sterols and good recovery for more and less retained oxides, a careful SPE procedure is needed. A common solvent mixture in phytosterol oxide purification has been hexane/diethyl ether with increasing polarity, as presented in Table 4. The oxide-containing fraction is often eluted with acetone. Since problems exist with elimination of unoxidized sterols, two-fold SPE and also other chromatographic techniques have been applied (Table 4). One approach has been to reduce the sample size and the amount of unsaponifiable lipids applied on an SPE cartridge (Lampi et al., 2002). When using silica cartridges, this carry-over effect can also be due to variable activities of sorbents. In this respect, even batch-to-batch variations have been observed (Guardiola et al., 2004).

Different chromatographic methods (column chromatography, preparative liquid chromatography, and TLC) have also been used to purify sterol oxides from lipid extracts without the preceding saponification step. The artifact formation and destruction of oxides occurring in the presence of an alkaline medium can thus be avoided; however, only sterol oxides formed from free sterols can be determined (Dutta et al., 1996; Guardiola et al., 2004).

2.3.2 Gas chromatographic determination

With efficient sample pre-treatment and by using capillary columns in GC separation at least some of the problems with the analysis of complex sterol oxide mixtures have been resolved. GC detection by flame ionization detector (FID) has provided rather good sensitivity and selectivity, and these properties have been further extended by using mass spectrometry (MS), especially in selected ion monitoring (SIM) mode. MS detection has diminished misidentification of sterol oxides in the presence of contaminants and coeluting compounds (Dutta et al., 1996; Guardiola et al., 2002). However, as can be seen in Table 4, the usage of MS in phytosterol oxidation studies thus far been rather limited.

Derivatization

In GC techniques, sterol oxides are usually analyzed as trimethylsilyl (TMS) ether derivatives due to their higher volatility and stability during separation at high temperatures (Dutta et al., 1996). Determination of underivatized oxides or acetate derivatives is also possible (Guardiola et al., 2004), but to overcome degradation, separation, and tailing problems or irreversible adsorption to the stationary phase of the GC column, TMS ethers are preferred (Fenton, 1992; Guardiola et al., 2004; Louter et al., 2004). With respect to MS detection, derivatization can also make electron ionization (EI) mass spectra easier to interpret due to less extensive fragmentation (Louter, 2004).

For derivatization of phytosterol oxides, different reagents have been used, as presented in Table 4. A common combination is hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS). Also a combination of *N,O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) is frequently used. The silanization is usually carried out in pyridine, which acts as a solvent and also as an HCl acceptor when silylation involves organochlorosilanes, such as TMCS (Guardiola et al., 2004). Anhydrous conditions are needed since water can compete with oxide hydroxyl groups (Guardiola et al., 2002). Other conditions, namely temperature and time, can also affect the strength of the reaction, especially in the case of triol derivatives of sterols (Dutta and Appelqvist, 1997). For instance, when using *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) for derivatization, bis-TMS ether of sitostanetriol was produced after 60 min of heating at 60°C. When derivatization was carried out at an ambient temperature overnight, tris-TMS ether derivative was formed (Zhang et al., 2005a).

Separation and detection

Fused silica capillary columns with nonpolar stationary phases (100% dimethyl polysiloxane or 5% diphenyl-95% dimethyl polysiloxane) and standard dimensions of 0.20-0.32 mm inner diameter and 25-30 m length are typically used in phytosterol oxide analytics (Dutta and Appelqvist, 1997; Bortolomeazzi et al., 2003; Lambelet et al., 2003; Apprich and Ulberth, 2004; Grandgirard et al., 2004a; Louter, 2004; Johnsson and Dutta 2005). Longer columns (50-60 m) have also been applied (Lampi et al., 2002; Conchillo et al., 2005; Zhang et al., 2005a,b).

The elution order of the main phytosterol oxides is identical in most of the above-mentioned studies: 7α-hydroxysterol, (unoxidized sterol), 7β-hydroxysterol, 5β,6β-epoxysterol, 5α,6α-epoxysterol, 3β,5α,6β-triol, and 7-ketosterol (Dutta and Appelqvist, 1997; Apprich and Ulberth, 2004; Grandgirard et al., 2004a; Zhang et al., 2005a). The series of campesterol derivatives is the first to elute, followed by stigmasterol and sitosterol derivatives (Bortolomeazzi et al., 2003; Apprich and Ulberth, 2004; Grandgirard et al., 2004a; Conchillo et al., 2005).

Although different stationary phases, column dimensions, and temperature programs have been used, problems still exist with the baseline separation and coelution. Dutta (1997) reported that baseline separation was achieved neither between the epimers of 5,6-epoxysterols nor between 7β-hydroxystigmasterol and 5α,6α-epoxycampesterol. Conchillo et al. (2005) found a poor separation between all 5β,6β-epoxysterols and some side-chain oxidation products, between 5β,6β-epoxysitosterol and campestanetriol, and between stigmastentriol and 5α,6α-epoxysitosterol. Coelution of 5α,6α-epoxysitosterol and campestanetriol (Apprich and Ulberth, 2004), 5α,6α-epoxystigmasterol and 7β-hydroxysitosterol (Lampi et al., 2002) as well as sitostanetriol and 7-ketocampesterol (Johnsson and Dutta, 2005) has also been noted. When Grandgirard et al. (2004a) examined gas chromatographic properties of several phytosterol oxides, also including many uncommon derivatives, coelution of, for instance, 7β-hydroxycampesterol and 6-hydroxycampestanol, 5β,6β-epoxycampesterol, and 4-campesten-6β-ol-3-one as well as 6-ketocampestanol and stigmastentriol was observed. In addition, a marked problem can be the coelution of unoxidized phytosterols with some phytosterol oxides, for instance, 7β-hydroxycampesterol and sitosterol (Lampi et al., 2002; Bortolomeazzi et al., 2003) or 7α-hydroxysitosterol and stigmasterol (Louter, 2004).

To resolve difficulties in GC separation, the combination of two capillary columns with different polarities (a non-polar 5% phenyl coated DB5-MS and a mid-polar 35% phenyl coated DB35-MS) has been tested. Enhanced baseline separation was, indeed, achieved, but three coeluting pairs were still observed: 24-hydroxysitosterol/5β,6β-epoxycampesterol, 5β,6β-epoxy-stigmasterol/5α,6α-epoxycampesterol, and 5α,6α-epoxystigmasterol/campestanetriol (Johnsson and Dutta, 2005).

Thus far, GC-MS has mainly been used in identification of sterol oxides, but because it also allows the identification and quantification of overlapping peaks, its use as a detector is increasing (Guardiola et al., 2002). SIM mode, especially, has advantages in sensitivity and selectivity due to exclusive acquisition of a specific ion or group of ions in a given time frame. Although FID has disadvantages when quantifying analytes in complex sample mixtures, it still is the main system for determining sterol oxides in foods since the FID response factors for sterol oxides are rather similar, i.e. close to 1.0, thus reducing the need for calibration for individual oxides (Guardiola et al., 2004; Louter, 2004).

Quantification

Generally, quantification of sterol oxides by GC is performed by the internal standard (ISTD) method (Apprich and Ulberth, 2004; Guardiola et al., 2004). As presented in Table 4, the most widely applied ISTD is 19-hydroxycholesterol, followed by 5α-cholestane and 7α-hydroxycholesterol. The use of 5α-cholestane has, however, several inconveniences. It has no hydroxyl function, i.e. it is not representative of oxidized sterols and as a nonpolar compound it must be added to samples only after purification steps since otherwise it will be lost in SPE (Grandgirard et al., 2004e; Guardiola et al., 2004). As most oxide losses seem to occur during saponification and extraction of unsaponifiable lipids (Lampi et al., 2002), the addition of an ISTD only prior to sample derivatization is problematic.

Problems also exist with 19-hydroxycholesterol: baseline separation may not be achieved if 7α-hydroxycampesterol is present, depending on the GC conditions (Grandgirard et al., 2004e). 19-hydroxycholesterol is otherwise regarded as a good choice: it has rather similar analytical behavior to sterol oxides and it can be added to samples prior to saponification, i.e. it compensates losses during the entire analytical procedure (Zhang et al., 2005a). Bortolomeazzi et al. (2003) used both 7-ketocholesterol and 7α-hydroxycholesterol as ISTDs to quantify the 7-keto and 7-hydroxyl derivatives of phytosterols, respectively. They suggested that when using 19-hydroxycholesterol more or less retained analytes in SPE could be lost with respect to the ISTD. Furthermore, they thought that 7-ketocholesterol better simulates possible decomposition of phytosterol oxides during saponification (Bortolomeazzi et al., 2003).

Table 4. Sample preparation when analyzing phytosterol oxidation products in foods by GC.

Food matrix	Lipid extraction	Internal standard	Saponification	Extraction of unsaponifiables	Isolation of phytosterol oxidation products	Derivatization	GC analysis	Reference
Potato chips	Hexane/ isopropanol	5α-cholestane added prior to derivatization	KOH in ethanol, at RT in the dark for 18 h	Dichloro-methane	Two-fold silica SPE 1. hex/dee 75:25 2. hex/dee 60:40 3. acetone *** 1. hex/dee 60:40 2. acetone	HMDS-TMCS in pyridine, at 60°C for 45 min	GC-FID with a falling needle injector	Dutta and Appelqvist, 1997
French fries	Hexane/ isopropanol	19-hydroxy-cholesterol added prior to saponification and 5α-cholestane added prior to derivatization	KOH in ethanol, at RT in the dark for 18 h	Dichloro-methane	Two-fold silica SPE 1. hex/dee 75:25 2. hex/dee 60:40 3. acetone *** 1. hex/dee 60:40 2. acetone	HMDS-TMCS in pyridine, at 60°C for 45 min	GC-FID with a falling needle injector	Dutta, 1997
Potato products	Hexane/ isopropanol	19-hydroxy-cholesterol added prior to saponification	KOH in ethanol, at RT in the dark for 18 h	Dichloro-methane	Two-fold silica SPE 1. hex/dee 75:25 2. hex/dee 60:40 3. acetone *** 1. hex/dee 60:40 2. acetone	HMDS-TMCS in pyridine, at 60°C for 45 min	GC-FID with a falling needle injector	Dutta and Appelqvist, 1996a
Coffee	Chloroform		-	-	Silica SPE 1. hex 2. hex/dee 80:20 3. hex/dee 50:50	-	GC-MS	Turchetto et al., 1993
Phytosterol ester-enriched spreads and fat blends	-	19-hydroxy-cholesterol added prior to saponification	KOH in ethanol, at RT in the dark for 18 h	Dichloro-methane	Normal phase LC using a gradient from 1.5% to 50% of isopropanol in hexane	HMDS-TMCS, in pyridine, at 65°C for 45 min	GC-FID, an on-column injector	Louter, 2004

Table 4. continued

Food matrix	Lipid extraction	Internal standard	Saponification	Extraction of unsaponifiables	Isolation of phytosterol oxidation products	Derivatization	GC analysis	Reference
Crude vegetable oils	-	7-ketocholesterol and 7α-hydroxy-cholesterol added prior to saponification	KOH in ethanol, at RT overnight	Diethyl ether	Silica SPE 1. hex/dee 1:1 2. dee/methanol 1:1 or aminopropyl SPE 1. hex/ethylacetate 95:5 2. hex/ethylacetate 9:1 3. acetone	HMDS-TMCS in pyridine, at RT for 1 h	GC-MS (ion trap) with a splitless injection mode	Bortolomeazzi et al., 2003
Wheat flour	Heptane/ isopropanol	7α-hydroxy-cholesterol added prior to saponification	KOH in methanol, at RT for 18 h	Diethyl ether	Lipidex-5000 using heptane/1,2-dichloro-ethane (19:1) as elution solvent, followed by aminopropyl SPE	HMDS-TMCS, at 60°C for 30 min	GC-FID with a falling needle injector	Nourooz-Zadeh and Appelqvist, 1992
Vegetable oil	-	7α-hydroxy-cholesterol added prior to saponification	KOH in methanol, at RT for 18 h	Diethyl ether	Lipidex-5000 using heptane/1,2-dichloro-ethane (19:1) as elution solvent	HMDS-TMCS at 60°C for 30 min	GC-FID with a falling needle injector	Nourooz-Zadeh and Appelqvist, 1992
Vegetable oil	-	5α-cholestane added prior to derivatization	KOH in ethanol, at RT in the dark for 16 h	Dichloro-methane	Silica SPE 1. hex/TBME 90:10 2. hex/TBME 80:20 3. acetone	BSTFA-TMCS in pyridine, at 60°C for 0.5 h	GC-FID with a falling needle injector	Lambelet et al., 2003
Phytosterol ester-enriched spread	Dichloro-methane/ methanol with 0.05% BHT	5α-cholestane added prior to derivatization	KOH in methanol, at RT under argon in the dark for 16 h	Dichloro-methane	Silica SPE 1. hex/TBME 90:10 2. hex/TBME 80:20 3. acetone	BSTFA-TMCS in pyridine, at 55°C for 0.5 h	GC-FID with a falling needle injector	Grandgirard et al., 2004a
Vegetable oils, Phytosterol ester-enriched spread	-	5α-cholestane added prior to derivatization	Transesterification with 10% sodium methylate in dry methanol diluted with MTBE	-	Two-fold aminopropyl SPE 1. hex/MTBE 2:1 2. acetone	HMDS-TMCS in pyridine, at 60°C for 45 min	GC-FID with an on-column injector	Johnsson and Dutta, 2006

Table 4. continued

Food matrix	Lipid extraction	Internal standard	Saponification	Extraction of unsaponifiables	Isolation of phytosterol oxidation products	Derivatization	GC analysis	Reference
Vegetable oil	-	19-hydroxy-cholesterol added prior to saponification	Saturated aqueous KOH solution, at 25°C in the dark overnight	Diethyl ether	Silica SPE 1. hex/dee 9:1 2. hex/dee 1:1 3. acetone	BSTFA-TMCS in pyridine, at RT overnight	GC-FID with an on-column injector	Lampi et al., 2002
Phytosterol ester-enriched spread	Chloroform/ methanol	19-hydroxy-cholesterol added prior to saponification	KOH in methanol, at RT in the dark for 18 h	Diethyl ether	Silica SPE 1. hex/dee 95:5 2. hex/dee 90:10 3. hex/dee 80:20 4. acetone	HMDS-TMCS in pyridine	GC-FID with a split-splitless injector (split ratio 1:30)	Conchillo et al., 2005
Vegetable oils, butter	-	19-hydroxy-cholesterol added prior to saponification	Saturated aqueous KOH solution, at RT in the dark for 15 h	Diethyl ether	Silica SPE 1. cyclohex/dee 9:1 2. cyclohex/dee 1:1 3. acetone	MSTFA in pyridine, at RT in the dark overnight	GC-MS with an on-column injector	Zhang et al., 2005a

BHT = butylated hydroxytoluene, BSTFA = *N,O*-bis-(trimethylsilyl)trifluoroacetamide, dee = diethyl ether, GC-FID = gas chromatograph with flame ionization detector, GC-MS = gas chromatograph – mass spectrometer, hex = hexane, HMDS = hexamethyldisilazane, KOH = potassium hydroxide, LC = liquid chromatography, MSTFA = *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide, MTBE = methyl *tert*-butyl ether, RT = room temperature, SPE = solid-phase extraction, TBME = *tert*-butylmethyl ether, TMCS = trimethylchlorosilane.

When an ISTD is used, the relative response factors (RRF) and linearity of response for each component should be established. Calibration of the GC system for the phytosterol oxide analyses is, however, a challenging procedure due to the absence of commercially available pure phytosterol oxides. For accurate calibration, individual phytosterol oxides should be synthesized. Considering the great number of oxides needed, these compounds are also prepared on a laboratory scale by heating phytosterols. This allows the formation of several oxides in one step (Grandgirard et al., 2004a). For instance, thermo-oxidation carried out at 135°C for 24 h has been observed to yield 7-hydroxy, 7-keto, 5,6-epoxy, 4β-hydroxy, 4-ene-6-hydroxy, and 6-keto derivatives (Grandgirard et al., 2004a). Oxidation at 150°C for 2 h yielded 7-hydroxy, 5,6-epoxy, and 7-keto derivatives (Conchillo et al., 2005).

Although no clear descriptions of how calibrations were carried out in phytosterol oxide determinations have been provided, it seems that in most studies the amount of phytosterol oxide has been calculated by comparing the peak area of the ISTD with a known concentration with the peak area of the oxide investigated (Dutta and Appelqvist, 1997; Dutta 1997; Louter, 2004; Conchillo et al., 2005; Johnsson and Dutta, 2006). In one study, GC-FID was calibrated with cholesterol oxides, and on the basis of the strong structural similarities between cholesterol and phytosterol oxides the same RRFs, 1.0-1.3, were used for quantification of phytosterol oxides (Apprich and Ulberth, 2004). Using the same idea, also the general RRF, 1.0, has been applied (Lampi et al., 2002).

Phytosterol oxides were synthesized and then used for calibration in only two studies. Bortolomeazzi et al. (2003) prepared 7α-hydroxy derivatives of sitosterol and campesterol and calculated RRFs of 0.854 and 0.857, respectively, in GC-MS when 7α-hydroxycholesterol was used as an ISTD. The same factors were used for quantification of 7β-epimers. For 7-ketositosterol, an RRF of 1.0 was assumed. Zhang et al. (2005a) synthesized 7-hydroxy, 5,6-epoxy, 7-keto and triol derivatives of sitosterol and calculated RRFs in relation to cholestanol or 19-hydroxycholesterol in GC-MS analysis. They found RRFs to be 0.62-1.99 with cholestanol and 0.86-2.73 with 19-hydroxycholesterol in a total ion chromatogram mode. In SIM mode, the respective values ranged between 0.12-6.44 and 0.16-8.29.

3 AIMS OF THE STUDY

When foods are enriched with food components, which normally occur in foods in low amounts, safety aspects of the procedure must be evaluated. The aim of this thesis was to produce scientific data to support the safety evaluation process of phytosterol/stanol-enriched functional foods in terms of stability. As phytosterols are lipid compounds, the main interest was in their oxidative stability. Using simplified food models and phytosterol/stanol-enriched foods, the susceptibility of phytosterols/stanols to oxidation in conditions present in food processing and storage was evaluated. As no official methods exist to determine phytosterol/stanol oxidation products in foods, a new method for their analysis was developed.

Detailed objectives were as follows:

1. To develop a gas chromatographic-mass spectrometric method for characterization and quantification of phytosterol/stanol oxidation products from complex food matrices (**I**).

2. To study factors affecting the oxidative stability of phytosterols/stanols in food models in order to identify critical conditions under which significant oxidation reactions may occur (**II, III**).

3. To examine the oxidative stability of phytosterols/stanols in enriched foods during processing and storage using food applications covering a range of commercially available phytosterol/stanol ingredients, different heat treatments during food processing, and different structures in multiphase foods (**IV, V**).

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4 MATERIALS AND METHODS

This section summarizes the materials and methods used in this work. More detailed information is presented in the original papers **I-V**.

4.1 Studies on phytosterol oxidation in food models (II, III)

4.1.1 Preparation of food models

Food models were prepared using tripalmitin (**II, III**) (Sigma, St. Louis, MO, USA, purity >85%) and RSO (**II**) (Raisio Plc., Raisio, Finland) as lipid matrices. Tripalmitin was enriched at a level equivalent to 1% sterol/stanol with either commercial sitosterol containing 55% sitosterol and 26% campesterol (**III**), stigmasterol (purity 95%) (**II**), or sitostanol (purity 95%) (**II**) (from Sigma), or with phytosterol or phytostanol esters (**III**) (from Raisio Plc.).

The phytosterol and phytostanol ester preparates used were mixtures and prepared by Raisio Plc. starting with soybean oil-derived sterols and RSO fatty acids. The main phytosterols in the sterol ester preparate were sitosterol (45%), campesterol (26%), and stigmasterol (15%), whereas the stanol ester preparate consisted of sitostanol (65%) and campestanol (33%). Expressed as percentages of total fatty acids, the respective proportions of total saturated, monounsaturated, and polyunsaturated fatty acids were 8%, 66%, and 26% in the sterol ester preparate, and 8%, 65%, and 28% in the stanol ester preparate (**III**).

In food model studies, phytosterol and stanol ester preparates as well as RSO were purified from pro- and antioxidants by adsorption chromatography using a glass column packed with activated aluminium oxide (Lampi and Kamal-Eldin, 1998). The lack of tocopherols after purification was checked by normal phase high-performance liquid chromatography (HPLC) using a silica column and fluorescence detection (Ryynänen et al., 2004).

4.1.2 Oxidation experiments

Oxidation experiments of enriched lipid samples (1.0 g) were conducted in glass vials (22 × 46 mm, i.d. 19 mm) in an oven using different temperatures and times as presented in Table 5. The vials were left open and shaken several times during heating. At each sampling point, the vials were taken from the oven and cooled in a desiccator before further analysis. All heating experiments were carried out three times, and each sample was analyzed in duplicate. To determine differences in phytosterol/stanol oxide formation between different heat treatments, between free and esterified phytosterols, and between sito- and campesterol, analyses of variance were carried out (Statgraphics Plus 4.0 software, Manugistics Inc., Rockville, MD, USA). In some cases, the data were not

normally distributed; therefore, a nonparametric Kruskal-Wallis test and a notched box-and-whisker plot were used. A confidence level of 95.0% was used in all statistical analyses.

Table 5. Oxidation experiments in food models.

Study	Lipid matrix	Phytosterol/stanol compound added[1]	Heating temperatures (°C) for all mixtures	Heating times for mixtures containing phytosterol compounds	Heating times for mixtures containing phytostanol compounds
II	Purified rapeseed oil[2]	Stigmasterol	60	1, 2, 3, 7 days	1, 2, 3, 7 days
		Sitostanol	100	3, 6, 24, 48 h	-
			140	0.5, 1, 3, 6 h	3, 6, 24 h
			180	0.5, 1, 2, 3 h	2, 3, 6 h
II	Tripalmitin	Stigmasterol	80	1, 2, 3, 4 weeks	2, 4, 8 weeks
		Sitostanol	100	3, 6, 24, 48 h	-
			140	0.5, 1, 3, 6 h	3, 6, 24 h
			180	0.5, 1, 2, 3 h	2, 3, 6 h
III	Tripalmitin	Sitosterol	100	3, 6, 24, 48 h	3, 6, 24, 48
		Campesterol	180	0.5, 1, 2, 3 h	2, 3, 6 h
		Purified phytosterol esters[2]			
		Purified phytostanol esters[2]			

[1]Lipid matrices were enriched with phytosterol/stanol compounds at a level equivalent to 1% sterol/stanol.
[2]Rapeseed oil and phytosterol and phytostanol esters were purified from pro- and antioxidants by adsorption chromatography.

Since the oxidative stability of saturated phytostanol compounds was predicted to be better than that of unsaturated phytosterol compounds, the heating experiments with stanol samples were fewer and more carefully thought out. Furthermore, because of the melting point (MP) of tripalmitin (66°C), heating of tripalmitin-based samples was started at 80°C instead of at 60°C.

4.2 Studies on phytosterol oxidation in enriched foods during processing and storage (IV, V)

4.2.1 Food matrices

Food applications selected for investigation were microcrystalline phytosterol suspensions in RSO, anhydrous milk fat (AMF), hydrogenated coconut oil (HCO), and refined palm kernel oil (RPKO) (**IV**), phytosterol-enriched whole milk powder (**IV**), phytosterol/stanol-enriched nonfat cow's milk (**IV**) as well as phytosterol/stanol-enriched rapeseed oil, butter oil, and liquid margarine (**V**). Phytosterol suspensions in different fats and oils were prepared by Teriaka Ltd. (Vantaa, Finland) using wood-based phytosterols (DRT, Dax Cedex, France). Two phytosterol concentrations in these suspensions were used: 18% and 30% (**IV**). Whole milk powder was also produced by Teriaka Ltd., with a microcrystalline phytosterol suspension in anhydrous milk fat serving as the phytosterol ingredient. The phytosterol content in the enriched milk powder was approximately 7% (**IV**).

Nonfat cow's milk was enriched with wood-based free phytosterols (from DRT) or with phytosterol or phytostanol esters (from Raisio Plc.) by MTT Agrifood Research (Jokioinen, Finland). Milks were enriched at a level equivalent to 0.4%, 0.5%, and 0.5% sterol/stanol, respectively (**IV**).

RSO, RSO-based liquid margarine (containing 80% fat) (from Raisio Plc.), and butter oil (Valio Ltd., Helsinki, Finland) were used as lipid matrices in pan-frying and were enriched with sitosterol (purity 75%) (Fluka Chemie, Buchs, Switzerland) or with phytosterol or phytostanol esters (from Raisio Plc.), at a level equivalent to 8% sterol/stanol (**V**). Since the pan-frying was a simulation of the actual cooking process, rapeseed oil and liquid margarine were not purified from pro- and antioxidants. To check the amount of α-tocopherol in these matrices, a method published by Nyström et al. (2007) was used. Briefly, normal phase HPLC was applied using a diol column and fluorescence detection.

4.2.2 Processing and storage conditions

Microcrystalline phytosterol suspensions in different fats and oils were prepared by heating the mixtures of fat/oil and phytosterols up to 100-110°C to form a clear solution and then cooled to about 90°C. During cooling boiled water was added and the suspensions were stirred until they reached RT. Suspensions were packed into 100-g polyethylene bags and stored in a refrigerator (4 ± 2°C) for 12 months. Phytosterol and phytosterol oxide concentrations in these suspensions were analyzed in triplicate at 3-month intervals (**IV**).

Phytosterol-enriched whole milk powder was produced by spray-drying. Heat treatments employed during this process were i) heating of the phytosterol ingredient to 70°C before emulsifying it to milk, ii) heating of the concentrated milk emulsion to 50-58°C after transferring it to a water jacket vessel, and iii) introduction of the milk emulsion to hot air, 165-192°C, in the drying chamber. Milk powder was packed into 20-g aluminum laminate bags and stored at RT (approximately 22°C) and slightly elevated temperature (38 ± 1°C) for 12 months. Powders were analyzed for phytosterol and phytosterol oxide concentrations in triplicate at 3-month intervals (**IV**).

To enrich nonfat cow's milk, free phytosterols or melted phytosterol/stanol esters were added to heated (70°C) milk also containing 0.4% distilled monoglycerides as an emulsifier. Milks were then homogenized and, to improve their durability, heated at 127°C for 2 s. Milks were packed into sterile 100-ml glass containers and stored in the dark at RT (approximately 20°C) and in a refrigerator (4 ± 2°C) for 6 months. Milks enriched with free phytosterols or phytosterol esters were analyzed for phytosterols and phytosterol oxides at 1.5-month intervals, and milks with phytostanol esters at 3-month intervals. Two batches of milks were stored, and analyses were performed in duplicate (phytosterol oxides) or triplicate (phytosterols) (**IV**).

Phytosterol-enriched RSO, liquid margarine, and butter oil were used as food applications to study the effect of pan-frying on phytosterol oxidation. For comparison, native rapeseed oil was also fried. An iron pan with an inner diameter of 21 cm was used for frying, and the sample size was 25 g of enriched oil/margarine. Fryings were performed at temperatures between 160°C and 200°C, and the frying time was 5-10 min. To equalize possible pro-oxidative effects caused by interactions between the iron pan and the thin film of oil, all fryings were randomized. Fryings were carried out twice, and each sample was analyzed for phytosterols and phytosterol oxides in duplicate (**V**).

4.3 Analysis of phytosterol oxidation products (I-V)

As no official methods exist to determine phytosterol oxidation products in foods, a new powerful method for their characterization and quantification in complex food matrices was developed. The actual sample preparation was, however, based on a method developed and validated earlier (Lampi et al., 2002).

4.3.1 Sample preparation

Sample preparation consisted of cold saponification, extraction of unsaponifiable material, purification of phytosterol oxides by SiOH-SPE, and derivatization to TMS ethers. In brief, cold saponification was conducted overnight at 25°C in the dark using saturated aqueous KOH solution. Extraction of unsaponifiable material was carried out with diethyl ether, after which the extract was washed with aqueous KOH and aqueous Na_2SO_4 and dried with anhydrous Na_2SO_4. Oxidized phytosterols were purified by SPE using hexane:diethyl ether (9:1, v/v) and hexane:diethyl ether (1:1, v/v) to remove unpolar compounds and unoxidized sterols, respectively, and acetone to elute phytosterol oxides. After drying under nitrogen, samples were subjected to overnight silylation by a BSTFA/TMCS (99:1, v/v) reagent in pyridine at RT. Before GC analysis, samples were evaporated and the residues were dissolved in hexane. All work was carried out under dimmed light conditions where possible.

In all samples, 19-OH-cholesterol (5-cholesten-3β,19-ol) (from Sigma or from Steraloids, Newport, RI, USA) was used as an ISTD and added prior to cold saponification. The amount of ISTD was adjusted depending on the expected oxide content of the sample.

4.3.2 Characterization and quantification

To be able to characterize and quantify a wide range of different phytosterol or phytostanol oxides in difficult food matrices during the same analysis, a new GC-MS method was developed. In terms of GC conditions, this method was partly based on material originally presented by Lampi et al. (2002). The applicability and specificity of the method developed was tested by quantifying

selected sitostanol oxides in purified RSO and tripalmitin, i.e. by having low levels of analytes in difficult matrices (**I**).

The equipment used was a Hewlett-Packard 6890 Series GC (Wilmington, PA, USA) coupled to an Agilent 5973 MS (Palo Alto, CA, USA). GC conditions selected were as follows: column: Rtx-5MS w/Integra Guard capillary column (crossbond 5% diphenyl - 95% dimethyl polysiloxane; Restek, Bellefonte, PA, USA) with film thickness 0.10 μm, 60 m × 0.25 mm i.d.; carrier gas: helium (>99.996%) at a constant flow of 1.2 ml/min; temperature program: 70°C (1 min), 40°C/min to 280°C (35 min). The injection technique used was an on-column injection. MS conditions selected were as follows: interface temperature 280°C; ion source 230°C; electron ionization (EI) energy: 70 eV. During the studies the performance of GC-MS was evaluated daily using a sterol standard mixture. Assessment of whether the GC separation was acceptable was based on the area ratio between cholesterol and dihydrocholesterol in the mixture. The mass spectrum of stigmasterol in this mixture was also monitored and evaluated daily.

Characterization of phytosterol and stanol oxides was done in full-scan mode (m/z 100-600). During the method development the GC-MS identification of phytostanol (mainly sitostanol) oxides was confirmed by TLC. In this procedure, analytical TLC plates (silica gel 60, 20 × 20 cm) (E. Merck, Darmstadt, Germany) were used, with heptane:ethyl acetate (1:1, v/v) acting as a solvent. Spots were visualized by staining with 10% H_2SO_4 in methanol. Visualized bands were scraped off the plate unstained, extracted with diethyl ether, filtered, and silylated for GC-MS analysis, as described in section 4.3.1.

GC-MS in SIM mode was used for quantification of identified phytosterol and phytostanol oxides, and for each compound selected for quantification, one target and 1-2 qualifier ions were chosen on the basis of their abundance and specificity for the compounds (Table 6). As no high-purity standards for individual phytosterol/stanol oxides are commercially available, the quantification of phytosterol/stanol oxides by the ISTD method was done indirectly, i.e. GC-MS was calibrated using phytosterol/stanol oxide mixtures prepared by thermal oxidation (phytosterols 180°C/1 h, phytostanols 180°C/3 h) and quantified by a previously published GC-FID method using a general RRF of 1.00 (Lampi et al., 2002). The use of this RRF was confirmed by commercially available structurally analogous cholesterol oxides (Lampi et al., 2002). To evaluate the day-to-day repeatability of the method applied, an in-house control sample (**I-III;** stigmasterol-enriched RSO oxidized at 180°C/3 h) was analyzed in each sample batch.

As GC-MS quantification was infrequently applied as a technique in phytosterol oxide studies, samples were simultaneously analyzed by GC-FID. The GC-FID consisted of a Hewlett-Packard 5890 Series II GC equipped with an automated on column injection system and an FID (Karlruhe, Germany). Conditions were as follows: Rtx-5 w/Intergra Guard capillary column (crossbond 5%

diphenyl - 95% dimethyl polysiloxane; from Restek) with film thickness 0.10 μm, 60 m × 0.32 mm i.d.; carrier gas: helium (>99.996%) at a constant flow of 1.4 ml/min; temperature program: 70°C (1 min), 60°C/min to 245°C (1 min), 3°C/min to 275°C (32 min); detector temperature: 300°C (Lampi et al., 2002).

Table 6. Target and qualifier ions for GC-MS-SIM quantification of phytosterol/stanol oxides (TMS ethers).

Study	Phytosterol/stanol oxide	Target ion (m/z)[1]	Qualifier ion(s) (m/z)[1]
I-V	19-OH-cholesterol (ISTD)	353	366 (M^+-2TMSOH)
II	7α/β-OH-stigmasterol	482 (M^+-TMSOH)	483
II	5α,6α/5β,6β-epoxystigmasterol	253	500 (M^+)
II	7-ketostigmasterol	359 (M^+-R')	498 (M^+)
III,V	7α/β-OH-campesterol	470 (M^+-TMSOH)	471
III,V	5α,6α/5β,6β-epoxycampesterol	488 (M^+)	398 (M^+-TMSOH)
III,V	7-ketocampesterol	486 (M^+)	381 (M^+-TMSOH-CH_3)
III-V	7α/β-OH-sitosterol	484 (M^+-TMSOH)	485
III-V	5α,6α/5β,6β-epoxysitosterol	502 (M^+)	412 (M^+-TMSOH)
III-V	7-ketositosterol	500 (M^+)	395 (M^+-TMSOH-CH_3)
I-V	7α-OH-sitostanol	486 (M^+-TMSOH)	487, 471[2] (M^+-TMSOH-CH_3)
IV-V	7β-OH-sitostanol	486 (M^+-TMSOH)	487, 471[2] (M^+-TMSOH-CH_3)
I-V	6α-OH-sitostanol	486 (M^+-TMSOH), 204[3]	576 (M^+), 205[3], 191[3]
IV-V	15α-hydroxysitostanol	269	270, 486[2] (M^+-TMSOH)
I-III	Unidentified compound of "RRT 1.779"[4]	486 (M^+-TMSOH)	576 (M^+)

[1]Decimals in target and qualifier ion values were used but are not presented since they varied slightly between calibrations. M^+, molecular ion; TMSOH, trimethylsilanol; R', side chain.
[2]During studies IV and V two qualifier ions were used to increase the specificity of the ions.
[3]Target and qualifier ions for 6α-OH-sitostanol were changed in studies IV and V to increase the specificity of the ions.
[4]Unidentified compound of relative retention time (RRT) 1.779 in relation to 19-OH-cholesterol.

To analyze the success of GC-MS quantification compared with GC-FID quantification, a paired-sample comparison was used (Statgraphics Plus 4.0). During studies the performance of GC-FID was evaluated daily using a sterol standard mixture as described above. In addition, an in-house control sample (**I-III;** stigmasterol-enriched RSO oxidized at 180°C/3 h or **VI**; cholesterol oxides in whole egg powder, stored at -70°C) was analyzed in each sample batch.

4.4 Other measurements (II-V)

4.4.1 Phytosterol contents in food models and foods

Phytosterol contents in unheated and heated samples were analyzed i) to establish the starting levels of phytosterols/stanols, ii) to be able to express the results as "oxidation percentages", i.e. as

percentages of total quantified oxides of the original unoxidized phytosterol/stanol, and iii) to study the oxidative stability of phytosterols/stanols not only through the formation of oxidation products but also through loss in initial phytosterol content.

Sample preparation was carried out using a previously published direct hot saponification method (Piironen et al., 2002). In brief, samples with dihydrocholesterol (3β-hydroxy-5α-cholestane; purity 95%) (from Sigma) as an ISTD were subjected to saponification at 85°C for 30 min using a saturated aqueous KOH solution. For extraction of unsaponifiables, water and hexane:diethyl ether (1:1) were added. Samples that contained ≤40% fat were extracted with cyclohexane. The extract was dried under nitrogen and subjected to silylation by BSTFA/TMCS (99:1, v/v) reagent in pyridine at 60°C for 30 min. Before GC-FID analysis, samples were evaporated and the residues were dissolved in hexane. GC-FID conditions were the same as described in section 4.3.2. Quantification was based on an ISTD method, and the amount of ISTD was adjusted to the expected amounts of phytosterols.

4.4.2 Measurements to study the oxidative behavior of lipid matrices

To clarify the overall changes that occurred in phytosterol-/stanol-enriched lipid matrices during heating, including possible interactions between the lipid matrix and phytosterols/stanols, the deterioration of lipid matrices was also evaluated in terms of polymerization and/or changes in unoxidized fatty acid moieties (**III, V**).

The formation of dimers and polymers was studied by high-performance size-exclusion chromatography (HPSEC), as described by Lampi and Kamal-Eldin (1998). Briefly, a Hewlett-Packard 1090 Series II HPLC and a 1047A refractive index detector were used (Waldbronn, Germany). Separation was performed on one 100-Å and two 50-Å PLGel columns (5 μm, 300 mm × 7.5 mm i.d.) (Polymer Laboratories Inc., Amherst, MA, USA) connected in series using dichloromethane at 0.6 ml/min as a mobile phase. Both the columns and the detector were thermostated at 35°C. The amounts of dimers and polymers were given as percentages based on peak areas, assuming equal responses of each lipid class. Tripalmitin, oxidized for 3 h at 180°C, was analyzed in each sample batch as an in-house reference sample (**III, V**).

Changes in unoxidized fatty acid moieties of the lipid matrix were studied as follows. With nonadecanoic acid (19:0) methyl ester (Nu Chec Prep Inc., Elysian, MN, USA) as an ISTD, the lipid samples were methylated using boron trifluoride (~10% in methanol) (Fluka Chemica, Buchs, Switzerland) and analyzed with GC. After GC analysis, fatty acid concentrations were calculated into triacylglycerols. Analyses were performed with a Hewlett-Packard 5890 GC equipped with an FID. The GC conditions were as follows: a fused-silica capillary column (NB-351; 0.20 μm, 25 m × 0.32 mm) (Hnu-Nordion Ltd., Helsinki, Finland); carrier gas: helium at 70 kPa; oven temperature:

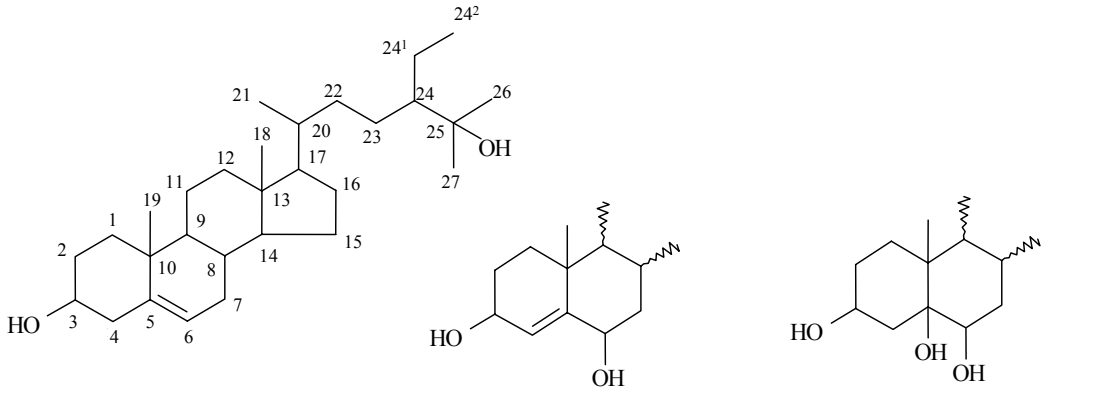
160°C (0.5 min), 5°C/min to 240°C (10 min); detector temperature: 260°C; injector temperature: 240°C. A split injection technique was used with a split ratio 1:28. RSO was analyzed in each sample batch as an in-house control sample (**III**).

5 RESULTS

5.1 Characterization and quantification of phytosterol oxidation products (I-V)

The mass spectral information achieved for phytosterol/stanol oxidation products with the GC-MS method developed was in accordance with data in the literature. In addition, the GC conditions used provided satisfactory resolutions for both phytostanol and phytosterol oxides, although baseline separation between all oxides was not achieved under these analytical conditions. Characterization of phytosterol oxide TMS ether derivatives with this method was based on mass spectral and GC properties previously reported in the literature (Dutta and Appelqvist, 1997; Lampi et al., 2002; Grandgirard et al., 2004a).

The main oxides characterized to form from sitosterol, campesterol, and stigmasterol during heating and/or storage experiments were 7α- and 7β-hydroxysterols, 5α,6α- and 5β,6β-epoxysterols, and 7-ketosterols. Only these oxides were quantified and thus used as markers of sitosterol, campesterol, and stigmasterol oxidation (**II-V**). In addition, traces of 25-OH-stigmasterol (**II**), 25-OH-sitosterol (**III, IV**), 25-OH-campesterol (**III**), 6α-OH-sitosterol (**V**), 6β-OH-sitosterol (**III, V**), 6β-OH-campesterol (**III**), 6-ketositostanol (**III-V**), 6-ketocampestanol (**III**), and 3β,5α,6β-sitostanetriol (**IV-V**) were detected and identified (Figure 4).



25-hydroxysitosterol 6-hydroxysitosterol 3β,5α,6β-sitostanetriol

Figure 4. Structures of some less common phytosterol oxidation products. 6-hydroxysitosterol can undergo epimerization to form both α- and β-epimers.

Characterization of oxides formed from phytostanols (mainly sitostanol) was challenging since literature in this area is scarce. Based on the mass spectral and GC information provided by Aringer and Nordström (1981), the following sitostanol oxides were tentatively identified: 7α- and 7β-OH-sitostanols, 15α- and 15β-OH-sitostanols, 6α-OH-sitostanol, 5β-OH-sitostanol, 7-ketositostanol, 6-ketositostanol, and 25-OH-sitostanol. To assure characterization, the mobility of these oxides on the TLC plates was also studied. In addition, all of the results were compared with those obtained for analogous derivatives of cholestanol (dihydrocholesterol) (**I**). Of the identified sitostanol oxides, 7α-OH-sitostanol and 6α-OH-sitostanol as well as one unidentified oxide of "RRT 1.779" (in relation to 19-OH-cholesterol in GC analysis) were selected as markers of sitostanol oxidation since their abundances were relatively high and they were well separated in GC (**I-III**). Based on the mobility of the unidentified oxide of "RRT 1.779" on TLC, it seemed to be a hydroxyl derivative of stanol ring structure, but in GC, it eluted just after 25-OH-sitostanol, revealing that it was instead a ketone or the hydroxyl derivative or the stanol side chain. In studies **IV** and **V,** the quantified oxides were 7α- and 7β-OH-sitostanols, 6α-OH-sitostanol, and 15α-OH-sitostanol, but 7-ketositostanol (**IV**-**V**) was also detected and identified (Figure 5).

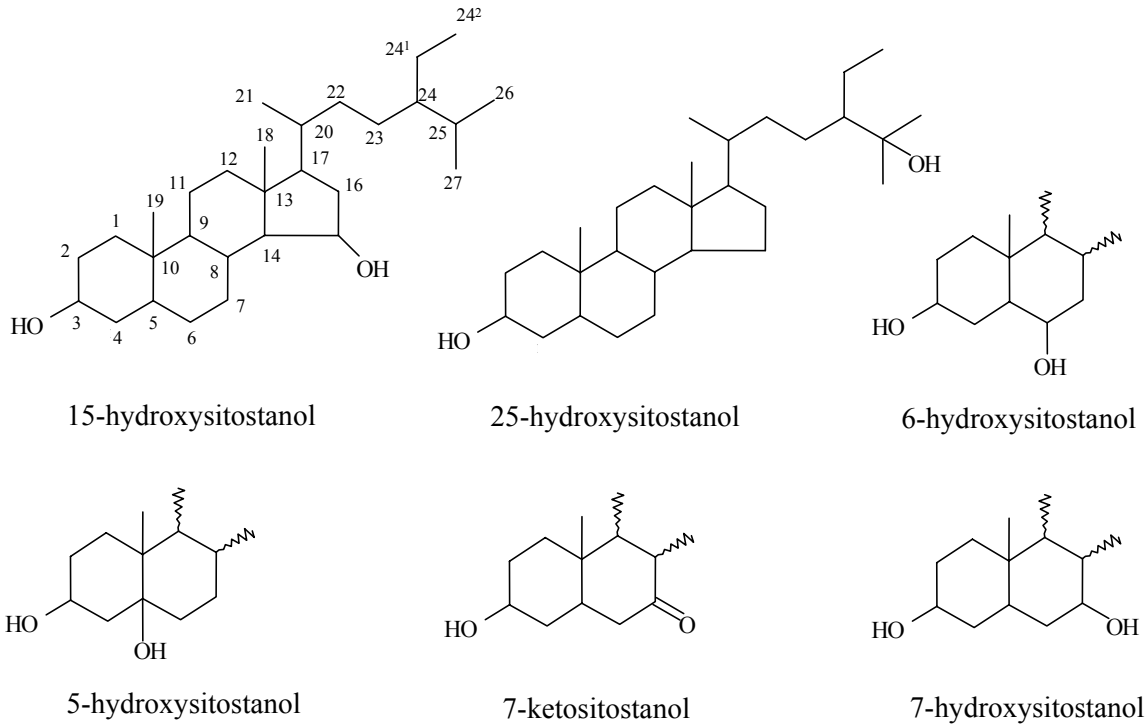


Figure 5. Structures of some phytostanol oxidation products. 15-, 6-, 5-, and 7-hydroxysitostanols can undergo epimerization to form both α- and β-epimers.

When constructing the GC-MS quantification, linear calibration curves for sitosterol, campesterol, stigmasterol, and sitostanol oxides were obtained. As an example, depending on the individual oxide, the ranges used were between 0.3 and 293 μg/g of lipid matrix for stigmasterol oxides and between 0.1 and 15.5 μg/g of lipid matrix for sitostanol oxides, the correlation coefficients (r^2) being 0.994-0998 and 0.991-0.997, respectively (**I, II**). The limits of determination we used were always set to the lowest levels of each calibration curve. Applicability of GC-MS quantification in phytosterol/stanol oxide studies was evaluated by comparing the results obtained by GC-MS with those obtained by GC-FID. This allowed us to note that the indirect construction of GC-MS calibration had only a minor effect on quantification. According to statistical analyses, depending on the oxide, the GC-MS results were the same or slightly higher than those quantified by GC-FID (**I, II**; Table 1). The coefficients of variation (CV) for oxide determination in heated samples by GC-MS were typically in the range of 0-20%. In comparing the GC-MS and GC-FID methods, it should be noticed that in some samples overlapping of ISTD with matrix compounds or coelution of different oxides unabled us to quantify with GC-FID.

5.2 Oxidative stability of phytosterols in food models (I-III)

In the next sections, all results concerning phytosterol and phytostanol oxidation are presented either as "oxidized phytosterol/stanol μg/g of lipid matrix" or as "percentages of phytosterol/stanol oxides of the original unoxidized phytosterol/stanol". Noteworthy is that oxidation of phytosterol/stanol esters here refers to the oxidation of sterol/stanol moiety in these molecules. During the sample preparation, specifically during saponification, esters were hydrolyzed and only the oxidized sterol moiety was analyzed. This was because our focus was on studying the phytosterol/stanol oxidation and factors (such as esterification) affecting it.

5.2.1 Effect of phytosterol structure

The oxidative stability of the sterol molecule was determined by the unsaturation degree of the ring structure. In all conditions studied, phytostanols were more stable than phytosterols, i.e. the presence of one double bond in the ring structure increased the susceptibility of phytosterols to oxidation (**II, III**). No differences were, however, observed in oxidation of sitosterol and campesterol which differ by one methyl group at carbon 24[1] (**III**). Furthermore, the oxidative behavior of stigmasterol as $\Delta^{5,22}$ -sterol was comparable with that of sitosterol and campesterol (**II**).

It should be noticed that comparison between oxidation percentages calculated for phytosterol- and stanol-enriched samples could be misleading since the oxides formed and their profiles are different for these compounds. However, by comparing the same kind of oxide (7α-OH) formed from stigmasterol and sitostanol, we observed that after heating a prolonged period (7 days) at moderate temperature (60°C) in a purified RSO matrix, the amount of 7α-OH-stigmasterol formed was 650

μg/g of matrix, while the amount of 7α-OH-sitostanol was 4 μg/g of matrix. The initial content of both oxides in unheated samples was <1 μg/g of matrix. Similarly, heating at high temperature (180°C) for 2 h resulted in the formation of 108 μg/g of matrix of 7α-OH-stigmasterol, while the corresponding stanol oxide amount was 2 μg/g of matrix (**II**; Tables 3 and 5). Analogous observations were made in saturated tripalmitin matrix: after 4 weeks of heating at 80°C, the amount of 7α-OH-stigmasterol formed was 174 μg/g of matrix, whereas the amount of 7α-OH-sitostanol was 0.4 μg/g of matrix (**II**; Tables 2 and 4).

The conjugation of the parent sterol structure, i.e. esterification with fatty acids, also had a significant effect on the oxidative stability of phytosterols, but in a rather complex way. In a saturated lipid matrix during prolonged heating at 100°C, esterification with RSO fatty acids made phytosterols more reactive than free sterols. At 180°C, however, the situation was the reverse, although the observed difference was quite small (**III**; Table 2). As an example, after 6 h of heating at 100°C, the amount of oxidized sitosterol and campesterol started to increase, reaching 16-17% after 48 h of heating. After the same heating time, only 1.2-1.3% of free sterols had oxidized. At a higher temperature, 180°C, while 22.4% of sitosterol had oxidized after 3 h of heating in an ester form, the oxidation percentage of free sitosterol was 26.5% (**III**; Table 2).

The oxidation reactions of phytostanol esters were low under all conditions studied and seemed to be less dependent on the structure of the stanol molecule (free vs. esterified). In a saturated lipid matrix after 6 h of heating at 100°C, oxidation of sitostanol in an ester form (0.001%) had not markedly proceeded compared with the initial state (0.001%). After the same heating time at 180°C, 0.5% of sitostanol in esterified form and 0.4% in free form had oxidized, indicating that the process had progressed similarly in both cases.

5.2.2 Effect of temperature and lipid matrix and their interactions

Oxidative stability of phytosterols and stanols was dependent on temperature. Generally, the oxidation reactions were more significant at temperatures above 100°C. At lower temperatures, the significance of these reactions was highly dependent on the reaction time. The lipid matrix was also shown to have a major role among the factors affecting the oxidation reactions. In addition, interactions between the sterol structure, matrix composition, and temperature led to interesting results in terms of oxidation susceptibility.

The comparison of stigmasterol oxide contents after heating for 3 h at 100°C, 140°C and 180°C revealed that phytosterols are oxidized at a much higher rate in conditions simulating deep-fat frying (180°C) than in conditions to which food might be subjected during other food processing (100°C /140°C). In tripalmitin, oxidation percentages of stigmasterol were 0.1%, 2.9%, and 24.2% and in purified RSO 0.8%, 3.2%, and 7.4% at 100, 140 and 180°C, respectively (**II**; Tables 2 and

3). The significance of heating time was revealed, especially at lower temperatures. As an example, virtually the same amount of oxides were formed from stigmasterol in tripalmitin matrix during 3 h of heating at 180°C and during 4 weeks (672 h) of heating at 80°C, i.e. 2268 and 2152 µg/g of matrix, respectively (**II**; Table 2). Despite the formation of sitostanol oxides being low at all temperatures and time points, the oxidation-accelerating effect of higher temperature and/or prolonged reaction time was also observed (**II**; Tables 4 and 5).

The effect of the lipid matrix (unsaturated vs. saturated) on phytosterol oxidation was rather paradoxical. At high temperatures (>140°C), sterols were more stable in an unsaturated matrix than in a saturated one. At lower temperatures (<140°C), this order was reversed. However, at 140°C, sterols oxidized almost at the same rate in both matrices. Illustrative examples of these phenomena were the oxidation percentages calculated for stigmasterol heated at 100°C for 48 h: 0.3% in tripalmitin and 26.5% in purified RSO – or at 180°C for 3 h: 24.2% in tripalmitin and 7.4% in purified RSO (**II**; Tables 2 and 3). When stigmasterol was heated in tripalmitin and rapeseed oil at 140°C for 6 h, the calculated oxidation percentages were 8.0% and 8.2% (**II**; Tables 2 and 3). Phytostanol oxidation was also dependent on lipid matrix composition, i.e. at all temperatures sitostanol was oxidized more in RSO than in tripalmitin, but in this case, no interactions between lipid matrix and heating temperature were observed (**II**; Tables 4 and 5).

To obtain tentative information on interactions between the lipid matrix and phytosterols/stanols, the deterioration of tripalmitin was also studied. In general, the oxidative changes observed in the matrix were not as great as in the phytosterols and were associated more with heating temperature and time than with phytosterol/stanol oxidation. Some interactions were, however, observed, at least with phytosterol esters: when phytosterol esters oxidized, the tripalmitin matrix slightly oxidized as well. In similar conditions, when phytostanol esters remained stable, no changes in tripalmitin were observed (**III**; Tables 1 and 2)

5.2.3 Oxidation product profiles

The distribution of quantified phytosterol and stanol oxides was monitored throughout the studies. While the interactions between the lipid matrix and heating temperatures affected the total oxide contents, they also had effects on the profile of the main secondary phytosterol/stanol oxides formed. Generally, the distribution of hydroxyl, epoxy, and keto compounds and changes in the proportions of 7-keto compounds, in particular, seemed to be associated with the phase of oxidation, i.e. whether the oxidation was in the lag or dynamic phase, and probably also how extensively other oxidation reaction pathways, such as polymerization, were involved.

7-ketosterols accumulated when oxidation had not yet reached the dynamic state. Once this state was attained, the major products were 5,6-epoxysterols and 7-hydroxysterols. This phenomenon

was observed for all phytosterols investigated whether they were in free or esterified form. As an example, in free sitosterol-enriched tripalmitin, the proportion of 7-ketositosterol at the beginning of heating was 37.1%, while the proportions of 7-hydroxysitosterols and 5,6-epoxysitosterols varied between 5.7% and 22.7%. After 3 h of heating at 180°C, the proportion of 7–ketositosterol was only 16.0%, but those of 7α-OH-sitosterol and 5α,6α-epoxysitosterol had increased being 23.8% and 25.5%, respectively. When sitosterol in esterified form was heated under the same conditions, even larger changes were observed: at the beginning of heating, the proportion of 7-ketositosterol was 41.7%, and 5β,6β-epoxysitosterol was not detected at all. After heating, the proportion of 7-ketositosterol decreased to 14.0%, while the proportion of 5β,6β-epoxysitosterol increased to 28.4% (**III**; Table 3).

Interactions between lipid matrices and temperature affected the profile of phytosterol oxides formed in a complex manner. When stigmasterol was heated in tripalmitin at 80°C/4 weeks or at 180°C/3 h and in purified RSO at 60°C/7 days or at 100°C/48 h, the overall oxidation percentages were similar, i.e. 23.0%, 24.2%, 23.2%, and 26.5%, respectively. As described above, the proportion of 7-keto derivates decreased and that of 7-hydroxides and 5,6-epoxides increased during heating of these samples – excluding the sample heated in tripalmitin at 80°C/4 weeks. For some reason, in this sample, the main oxide throughout the treatment was 7-ketostigmasterol. Another illustrative example of the effect of lipid matrix was heating of stigmasterol in tripalmitin and RSO at 140°C for 6 h. The percentages of overall oxidation were almost the same in these samples, approximately 8%, and the main product at the beginning of heating was 7-ketostigmasterol. After 6 h of heating in tripalmitin, 7-ketostigmasterol remained the main product, but in rapeseed oil its proportion had clearly decreased and was the lowest of all (**II**; Tables 2 and 3).

The product profile of sitostanol also changed during heating both in the case of free and esterified stanols. In purified RSO, the proportion of the unidentified oxide of "RRT 1.779" was the highest at all heating temperatures (60-180°C) studied. In tripalmitin, at 80°C, the product profile was similar to that in RSO. However, during heating at 100°C, 140°C and 180°C, the proportion of unidentified oxide of "RRT 1.779" decreased and those of 6α-OH-sitostanol and 7α-OH-sitostanol increased (**II, III**).

5.2.4 Formation of previously uncharacterized phytosterol oxidation products

In addition to measurements of phytosterol/stanol oxides formed, loss of initial phytosterol content was investigated. By combining these results, we noticed that the formation of secondary oxides did not account for all phytosterol losses observed after heating. Instead, there was a significant "gap" between them. At lower temperatures, this "gap" was small, i.e. the formation of quantified secondary oxidation products explained the losses observed in the initial phytosterol/stanol

contents. At higher temperatures, however, quantified oxides accounted for a lower proportion of sterol/stanol loss, indicating that other oxidation reactions were also occurred. Moreover, the extent of these other reactions at high temperatures seemed to be different for free and esterified phytosterols/stanols. In the case of free sterols/stanols, the "gap" was significantly larger (**III**).

As presented in Figure 6, for free and esterified phytosterols (sitosterol), the "gap" was smallest when heated for 48 h at 100°C, being 1.4% and 2.8%, respectively. For comparison, the corresponding "gaps" when these samples were heated for 3 h at 180°C were 27.5% and 12.7%.

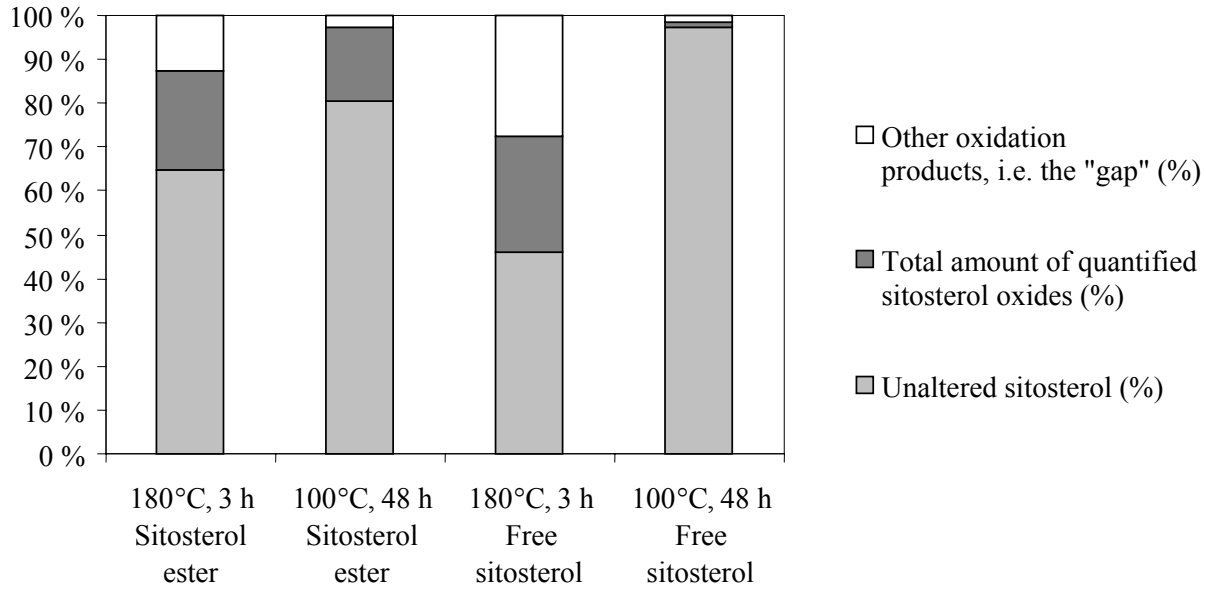


Figure 6. Percentages of unaltered sitosterol, quantified secondary oxides, and unknown oxidation products after two different heating experiments. Free and esterified sitosterols (1%) were heated in a tripalmitin matrix.

For esterified phytostanols (sitostanol), no losses in initial stanol content were noted when heated at 100°C for 48 h. For free and esterified sitostanols, after 6 h of heating at 180°C, the "gaps" were as large as 41.1% and 10.9%, respectively (Figure 7) (**III**; Figure 4). Noteworthy is that in addition to secondary oxides, 7α-OH-sitostanol, 6α-OH-sitostanol, and the unidentified oxide of "RRT 1.779" quantified by GC-MS, other secondary oxides (see section 5.1), originating from the polar SPE fraction and detected by GC-FID, were included in these illustrations. The oxides quantified by GC-MS (and used as markers of sitostanol oxidation in our studies) constituted only approximately 10% of the compounds in that fraction.

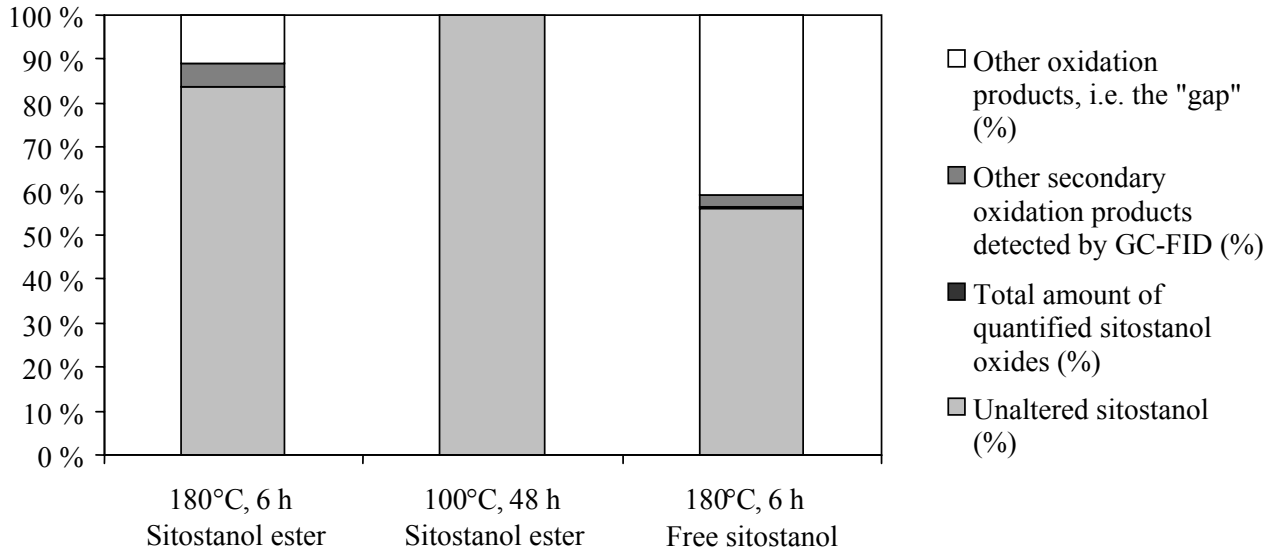


Figure 7. Percentages of unaltered sitostanol, quantified secondary oxides, other secondary oxides, and unknown oxidation products after two different heating experiments. Free and esterified sitostanols (1%) were heated in a tripalmitin matrix.

5.3 Oxidative stability of phytosterols in foods (IV, V)

In the next sections, all results concerning phytosterol and phytostanol oxidation are presented either as "oxidized phytosterol/stanol μg/g of lipid matrix" or as "percentages of phytosterol/stanol oxides of the original unoxidized phytosterol/stanol at that time point". As described in section 5.2, phytosterol/stanol esters refer to the oxidation of sterol/stanol moiety in these molecules.

5.3.1 Cold storage of microcrystalline phytosterol suspensions in different fats and oils (IV)

Microcrystalline phytosterol suspensions in RSO, AMF, HCO, and RPKO were stable during the 12-month storage at 4°C. At any given time point, the amount of quantified phytosterol oxides was virtually the same as at the beginning of storage, ranging from not determined to 54 μg/g of product in suspensions containing 18% phytosterols and from 27 to 109 μg/g of product in suspensions containing 30% phytosterols. The highest percentages of oxidation, 0.04-0.05%, were observed in RSO suspension containing 30% phytosterols. The lowest proportions were in RSO suspension containing 18% phytosterols (0.01-0.02%) and in HCO suspension containing 30% phytosterols (0.01-0.02%) (**IV**; Tables 1 and 2).

At the beginning of storage, slight differences in total oxide contents were observed between different matrices and phytosterol contents (18% vs. 30%). The above-mentioned finding that no increase in the initial oxide contents could be measured during the one-year storage indicated that these initial differences were, at least, partly derived from the phytosterol preparations used in the

enrichments. However, processing may also have caused some phytosterol oxidation depending on the lipid matrix. On the other hand, because the measured phytosterol contents were so close to added levels (18% and 30%), the effect of processing appears to be negligible.

The main phytosterol oxides quantified in the above-mentioned suspensions were 7β-OH-sitosterol and 5α,6α- or 5β,6β-epoxysitosterols. The amounts of 7α-OH-sitosterol and 7-ketositosterol were slightly lower or remained under the determination limits set (5 μg/g of product for 7-hydroxysterols and 5,6-epoxysterols; 10 μg/g of product for 7-ketosterols). 25-OH-sitosterol and 6-ketositostanol were also detected, and, interestingly, the latter seemed to be strongly characteristic to wood sterol preparation used in enrichment (see section 4.2.1). Its amount did not increase during storage, but its initial amount was higher than that of other oxides.

5.3.2 Heat treatment and subsequent storage of phytosterol/stanol-enriched milks (IV)

Nonfat cow's milks enriched with free or esterified phytosterols (sitosterol) or with phytostanol (sitostanol) esters were observed to be stable during the 6-month storage at RT and at 4°C when compared with the initial state of oxidation. At the beginning of storage, the oxidation percentages in these milks were 0.08%, 0.09% and 0.01%, respectively. After 6 months of storage, the corresponding oxidation percentages were 0.07%, 0.10% and 0.01% at RT and 0.07%, 0.11% and 0.01% at 4°C. Slight decreases and increases in oxide contents were mainly due to low oxide levels causing rather large standard deviations. However, differences could partly be due to sterol structures. Sitostanol seemed to be more stable than free sitosterol, which in turn seemed to be more stable than esterified sitosterol (**IV**; Table 4).

Of the quantified oxides, the main phytosterols in milks were 7α- and 7β-OH-sitosterols and 7-ketositosterol. However, at the beginning of storage, the amount of 6-ketositostanol was the highest, as also observed in microcrystalline phytosterol suspensions (see section 5.3.1). In phytostanol-enriched milks, the only sitostanol oxide found above the determination limits (ranging between 0.01-0.06 μg/g of product for quantifiable sitostanol oxides) was 6α-OH-sitostanol. The use of only 6α-OH-sitostanol as a marker of sitostanol oxidation in milks was, however, a bit controversial since we also gave evidence that this oxide may have been derived from the rapeseed oil used in the esterification process of the phytostanol ingredient.

Although no significant changes were observed in phytosterol/stanol oxide contents during storage experiments, the heat treatment (127°C for 2 s) applied during milk processing appeared to slightly enhance sitosterol oxidation. This was deduced from oxidation percentages in raw materials used in enrichments; in free sitosterol and in phytosterol and phytostanol ester preparates the percentages were 0.04%, 0.02%, and 0.01%, respectively. When these proportions were compared with those calculated after processing and subsequent storage, two-fold (0.07-0.08%) or even five-fold (0.09-

0.11%) percentages were observed for free and esterified sitosterols, respectively. For sitostanol ester, no change in oxidation percentage during storage was calculated compared with that of raw material.

5.3.3 Spray-drying and subsequent storage of phytosterol-enriched milk powders (IV)

Phytosterols were stable in whole milk powder during the 12-month storage period at RT. At a slightly elevated temperature (38°C), phytosterols oxidized more rapidly. At RT the content of sitosterol oxides increased from 14 to 19 μg/g of product and at 38°C from 14 to 34 μg/g of product. These contents correspond to oxidation percentages of 0.03-0.04% at RT and 0.03-0.07% at 38°C (**IV**; Table 3). The accumulation of quantifiable sitosterol oxides consisted of only 7α- and 7β-OH-sitosterols. At the beginning of storage, the main oxide was 6-ketositostanol, as also observed also in microcrystalline phytosterol suspensions and enriched milks. This is because the same wood sterol preparation was used for enrichment of all of these foods. In addition to the above-mentioned oxides, 25-OH-sitosterol and 3β,5α,6β-sitostanetriol were detected in stored milk powders.

When differences in oxide amounts were studied through changes in initial phytosterol contents, no alteration was observed; at the beginning of storage, the phytosterol content in milk powders was 6.9 g/100 g and after 12 months of storage 6.6 g/100 g both at RT and elevated temperature. The effect of spray-drying was also minor: the oxidation percentage after production of milk powder was 0.03%, being thus in the same range with oxidation percentages calculated for raw materials used in enrichment (phytosterols in AMF) (**IV**; Tables 1 and 2).

5.3.4 Pan-frying of phytosterol-enriched lipid matrices (V)

Pan-frying at high temperatures seemed to induce phytosterol oxidation but had no marked effect on phytostanol oxidation; up to 5.1% of initial sitosterol and 0.1% of initial sitostanol was found as oxides. In general, the higher the frying temperature and the longer the frying time, the more phytosterols oxidized. In addition, a saturated lipid matrix (butter oil) and the presence of water in the lipid matrix (liquid margarine) accelerated phytosterol oxidation. This study also showed that during frying phytosterol oxidation proceeded similarly in native and enriched rapeseed oils. As an example, at 180°C, after 0, 5, and 10 min of frying, 0.1%, 0.7%, and 1.7% of indigenous sitosterol, 0.0%, 0.5%, and 1.8% of added free sitosterol, and 0.1%, 0.5%, and 1.8% of added sitosterol esters were oxidized, respectively, when expressed as “percentages of phytosterol/stanol oxides of the original unoxidized phytosterol/stanol” (**V**; Table 1).

As presented in Figure 8, the highest phytosterol oxidation percentages were quantified in butter oil, followed by liquid margarine and RSO. Since the fatty acid compositions and the amount of α-tocopherol were rather similar for RSO and RSO-based liquid margarine, the presence of water in

the matrix seemed to play an important role in terms of phytosterol oxidation during frying. When comparing the oxidation susceptibility of free and esterified sterols in these matrices, the former were observed to oxidize more than the latter, especially in liquid margarine and butter oil.

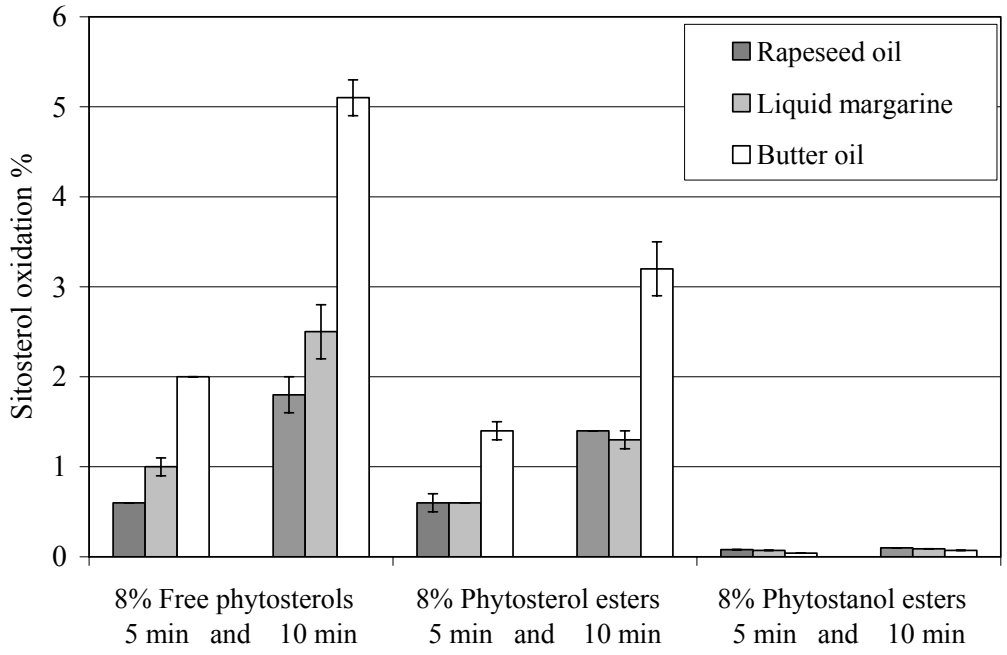


Figure 8. Sitosterol oxidation (%) in phytosterol/stanol-enriched lipid matrices during pan-frying at 180°C.

The spectrum of identified oxides formed in phytosterol-enriched pan-fried lipid matrices was larger than observed in other food applications. In addition to the quantified main oxides, 7-hydroxides, 5,6-epoxides, and 7-ketones, small amounts of 6-ketositostanol, 6α- and 6β-OH-sitosterols, 6α-OH-sitostanol, 6α-OH-campestanol, 3β,5α,6β-sitostanetriol and some stigmasterol and brassicasterol oxides were detected. Of the quantified oxides, the proportion of 7-ketositosterol decreased during frying and the proportion of especially 7β-OH-sitosterol increased. The higher the frying temperature, the faster these changes appeared to happen.

In phytostanol-enriched pan-fried lipid matrices, 7α-, 7β-, 6α-, and 15α-OH-sitostanols were quantified, but 7-ketositostanol was also detected. As described in section 5.3.2, the use of 6α-OH-sitostanol as a marker of oxidation here might be somewhat controversial since it may have been derived from the rapeseed oil used as a matrix. This line of though was strengthened by observations that 6α-OH-sitostanol was also present in rapeseed oil-based samples enriched with phytosterols. However, since the amount of this oxide increased further during frying in stanol-enriched samples, it was still used as one marker of stanol oxidation.

To obtain preliminary information on interactions between lipid matrices and phytosterols/stanols, the deterioration of matrices was also studied in terms of polymerization. HPSEC determinations revealed a moderate degree of dimer and polymer formation. The extent of these reactions was mainly determined by frying temperature and time, i.e. the higher the temperature and the longer the frying time, the more lipid matrices polymerized. As shown in Figure 9, unsaturation degree and water content of the lipid matrix also played an important role; the more unsaturated the matrix and the more water it contained, the more it polymerized.

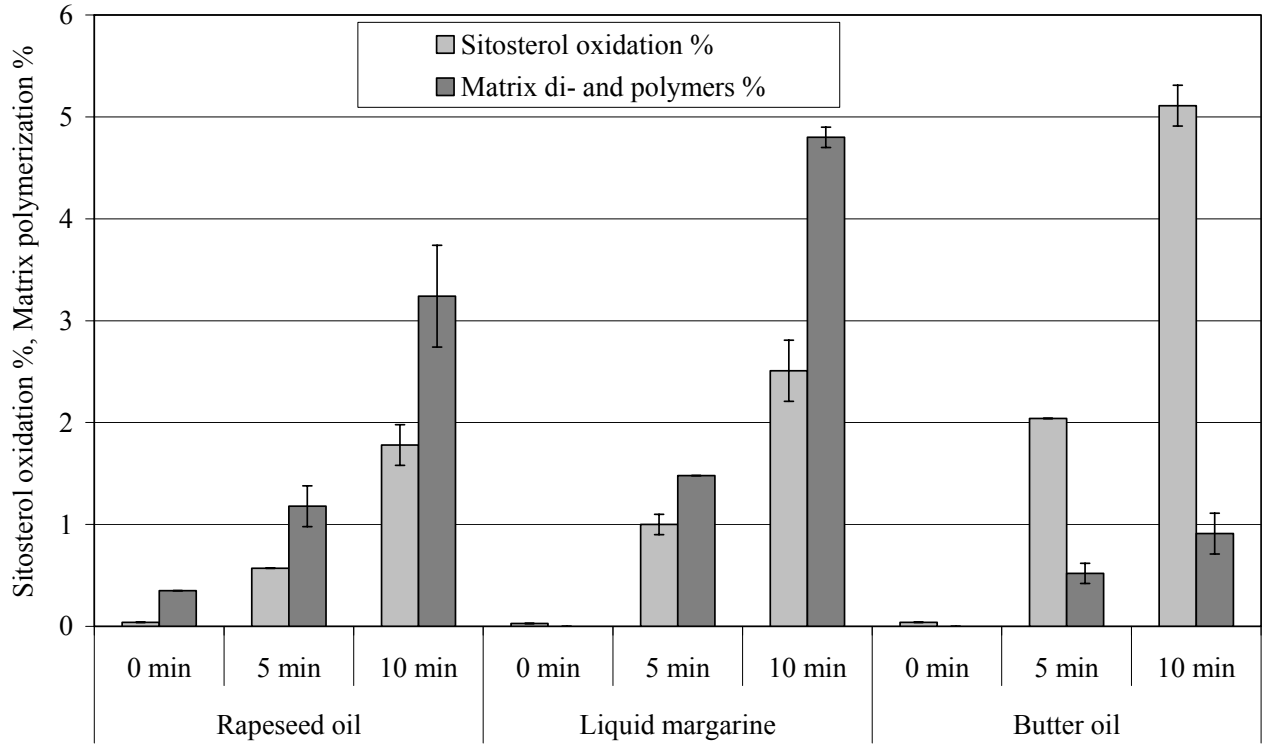


Figure 9. Formation of phytosterol oxidation products (%) and matrix dimers and polymers (%) in free phytosterol-enriched (8%) lipid matrices during pan-frying at 180°C.

In addition to the high susceptibility of liquid margarine to polymerization, other interesting observations were made in this matrix. In contrast to the other matrices and temperatures studied, liquid margarine polymerized differently during frying at 180°C, depending on the phytosterol compound used in enrichment. The more the phytosterol compound oxidized, the more the liquid margarine appeared to polymerize (**V**; Table 3). Considering these kinds of interactions, we also observed that at 160°C, phytosterols and stanols seemed to slightly inhibit RSO polymerization. In native RSO, the dimer and polymers content were 0.4%, 0.8% and 1.5% after 0, 5, and 10 min of frying and in phytosterol ester-enriched RSO, the respective contents were 0.5%, 0.5%, and 0.6% (**V**; Table 1).

6 DISCUSSION

6.1 Evaluation of the gas chromatographic-mass spectrometric method

A new GC-MS method for characterization and quantification of phytosterol and phytostanol oxides was developed. Because of the high specificity and selectivity of this method, we were able to quantify low amounts of different phytosterol/stanol oxides in more complex food matrices than possible with the more commonly used GC-FID method. This work thus contributed to knowledge of new techniques suitable for phytosterol/stanol oxide analytics in foods.

Thus far, GC-MS has been used for phytosterol oxide quantification in only a few studies (Turchetto et al., 1993; Plat et al., 2001; Bortolomeazzi et al., 2003; Zhang et al., 2005a). In the absence of commercial standards for individual phytosterol oxides, calibration has been performed using structurally analogous cholesterol oxides (Turchetto et al., 1993) or by synthesizing phytosterol oxides on a laboratory scale (Plat et al., 2001; Bortolomeazzi et al., 2003; Zhang et al., 2005a). The latter, although being the most accurate method, is a laborious procedure considering the great number of phytosterol oxides to be prepared and the complex stereochemistry of phytosterols. One problem that also exists in laboratory-scale synthesis is the lack of pure commercial phytosterols as starting materials. The use of phytosterol blends results in the formation of oxide mixtures of different phytosterols during synthesis (Plat et al., 2001; McCarthy et al., 2005). The purification of individual phytosterols from blends is also a time-consuming project; by using a semi-preparative reversed phase HPLC technique, at least 51 cycles were reported to be needed to obtain 79 mg of pure sitosterol and 12.3 mg of pure campesterol (Grandgirard et al., 2004a). By using preparative silica gel adsorption chromatography, 8-10 cycles yielded 2 g of sitosterol, but still 2-5% of campesterol was present (Zhang et al., 2005b). Furthermore, as far as the actual oxide syntheses are concerned, the yields seem to be variable and modest (Johannes and Lorenz, 2004; McCarthy et al., 2005).

Since prerequisites for the development of methods for phytosterol oxide determinations in foods are difficult to conform, literature regarding phytosterol oxide analytics and the presence of oxides in foods is scarce. A discrepancy exists since the introduction of phytosterol-enriched foods into human nutrition necessitates these studies. In the present study, efforts were made to produce scientific data to support the safety evaluation process of phytosterol/stanol-enriched foods in terms of the oxidative stability of added phytosterols/stanols. This goal was achieved by applying an indirect quantification method for phytosterol/stanol oxide determination by GC-MS. The method was classified as indirect since phytosterol oxides for calibration were prepared by thermo-oxidation and the oxide mixtures formed were quantified by GC-FID using an RRF of 1.00 (19-OH-cholesterol as ISTD). The use of this RRF was based on the average RRF calculated for hydroxy, epoxy, ketone, and triol derivatives of cholesterol (Lampi et al., 2002). The quantitative

information yielded by GC-FID was then used for calibration of GC-MS with these oxide mixtures. Metrologically, the more accurate method naturally is one based on synthesized phytosterol oxides, but the much higher costs and efforts involved necessitate compromises being made.

The idea of using indirect calibration was supported by a recent study by Apprich and Ulberth (2004). As in our study, they first calibrated the FID system with cholesterol oxides and based on the strong structural similarities made the assumption that both phytosterol and cholesterol oxides have the same FID responses (19-OH-cholesterol as an ISTD, column type HP-5). Phytosterol oxides quantified by this procedure were then used to calculate full scan mode MS response factors. The resulting RRFs for phytosterol oxides agreed with those of gravimetrically prepared cholesterol oxides, which in turn agreed with those calculated for FID; all were within 0.99-1.35 (Apprich and Ulberth, 2004). The conclusion was drawn that the chromatographic and mass spectrometric behavior of phytosterol and cholesterol oxides was sufficiently similar to allow calibration of the GC-MS system to be done via GC-FID. Noteworthy, however, is that when using GC-MS in SIM mode the RRFs can be highly variable (Zhang et al., 2005a). Thus, RRFs for individual phytosterol oxides should always be checked and not assumed to be of 1.00 in GC-MS.

Since the sample preparation and the GC-FID method used in this study were developed and extensively validated earlier by Lampi et al. (2002), the validity of the GC-MS method developed here was mainly checked by comparing the phytosterol oxide amounts obtained by GC-MS with those obtained by GC-FID. In both phytosterol and phytostanol oxide studies, depending on the oxide, the GC-MS results were the same or slightly higher than those quantified by GC-FID. The differences observed were, however, considered insignificant when compared with, for instance, interlaboratory studies of cholesterol oxides in foods having CV values of up to 240% for individual oxides, despite harmonized methods being used (Dutta and Savage, 2002b). The differences observed in our study were largely due to the too low or high amounts of ISTD compared to the amounts of analytes in our samples. Since variable amounts of five different phytosterol oxides or 3-4 different phytostanol oxides were quantified in the same GC-MS run, the optimal ISTD addition was sometimes difficult to achieve. Another point to be highlighted when using GC-MS is that the selection of characteristic target ions for quantification needs to be done with care. Due to the presence of many different oxides in the same sample as well as the possible presence of unoxidized phytosterols or some other impurities, only the high specificity and selectivity of ions for the compound guarantees the success of quantification. In our study, we increased the accuracy of ions for quantification by using 1-2 qualifier ions in addition to one target ion.

The challenges of using GC-MS quantification may raise a question about the desirability of MS detection compared with the more commonly used FID. In routine analyses, the GC-FID is still the easiest method for phytosterol oxide determinations. However, our study clearly showed that when phytosterol oxides are determined in complex food matrices, an effective purification system is not

always sufficient, and thus, difficult analytical situations may arise when using FID as a detector. In our study, the overlapping of the 19-OH-cholesterol as an ISTD with compounds from the rapeseed oil matrix made the use of FID for quantification impossible. Furthermore, in the case of phytosterol-enriched milks and milk powders, some unknown impurities eluted in GC-FID analyses and interfered with the quantification of phytosterol oxides.

6.2 Critical conditions for oxidative stability of phytosterols

Role of food models

To improve understanding of factors determining oxidative behavior of phytosterol/stanol compounds in foods, the food model studies were conducted. The known composition of the sample evaluated and strictly controlled external parameters produced general simplified information, which was also of use when estimating the stability of phytosterols in foods. In our food model studies, RSO and tripalmitin were selected as lipid matrices. Since phytosterols and stanols as lipid compounds are in close proximity to other lipids in foods, the use of lipid models was relevant. RSO was regarded as a good representative of an unsaturated lipid matrix because it is widely used as a food oil and is already applied in commercial phytosterol/stanol-enriched foods. To emphasize the effect of the RSO triacylglycerol composition in oxidation, the natural anti- and pro-oxidants were removed. Tripalmitin represented a common triacylglycerol present in foods containing saturated fat (Labuza, 1971). Initially, the use of a saturated lipid matrix in food models was regarded as informative since its less active participation in oxidation reactions was thought to make interpretation of phytosterol/stanol oxidation easier.

Sitosterol, campesterol, and stigmasterol were used as representatives of phytosterols since they are the most commonly encountered phytosterols in higher plants (Akihisa et al., 1991; Moreau et al., 2002), and thus, in phytosterol-enriched foods. Sitostanol, which was also studied, is the main phytostanol in natural sources in the human diet (Dutta and Appelqvist 1996b; Piironen et al., 2002) as well as in phytostanol-enriched foods (Salo et al., 2005). Since both free and esterified phytosterols and stanols are used in production of novel foods, both were also investigated here.

Temperatures, 60-180°C, and reaction times, 0.5 h – 8 weeks, used in food model studies simulated conditions that phytosterols/stanols encounter during food processing (cooking, baking, roasting, deep-frying, etc.). The lowest temperature studied, 60°C, was also of use when modeling the oxidative stability of phytosterols during storage at ambient temperatures. At temperatures above 60°C, the hydroperoxides formed decompose and the number of side reactions (such as polymerization) increase, and thus, the results do not correlate well with evaluations of actual shelf-life of lipid-containing foods (Frankel, 1993; 1998). Drastic heating was also carried out in order to identify critical conditions under which significant oxidation reactions occur.

To increase reliability of the results, all heating experiments were carried out three times and each sample was analyzed in duplicate. Considering that the experimental data were influenced both by the methodology used and by the nature of lipid oxidation, the CV values observed (mainly <20%) were in good agreement with accepted CVs for analytical methods working in the μg/g-region (Horwitz et al., 1980). These CVs also revealed that although lipid oxidation as a complex phenomenon is difficult to control, especially under slow oxidation and during prolonged stability tests (Frankel, 1993), the experiments in our study were conducted properly.

Major factors affecting phytosterol/stanol oxidation

The data obtained in food model studies revealed that the oxidative stability of phytosterols, measured as the total amount of the main secondary oxides formed, and the oxidation product profile depended on the sterol structure, reaction temperature, reaction time, and matrix composition. Phytostanols were observed to be rather stable and their reactions were mainly associated with heating temperatures and times. One of the major findings was that especially at higher reaction temperatures the secondary oxides used as a marker of phytosterol and stanol oxidation here did not account for all sterol/stanol losses observed. New important information was thus yielded about phytosterol/stanol oxidation, which has up till now been studied relatively little.

The differences in oxidation susceptibility between unsaturated (sterols) and saturated (stanols) phytosterols indicates that the oxidation of sterol compounds follows the same chemistry as the oxidation of other lipids; the rate of oxidation increases with increasing number of double bonds (Porter et al., 1995). It is important, however, to recognize that in the present study the oxide patterns used as markers of oxidation for phytosterols and phytostanols were different, and thus, no exact comparison could be made. We did compare the formation of the same kind of oxide, 7α-hydroxy derivative, from phytosterols and stanols but since the oxidation mechanism of saturated lipids may differ from that of unsaturated lipids (Swern, 1961), the conclusions drawn are only tentative. However, when the oxidative stability of sitostanol, stigmasterol, and ergosterol heated in mineral and rapeseed oils was studied through losses in initial sterol/stanol content, sitostanol, indeed, was the most stable and ergosterol with three double bonds ($\Delta^{5,7,22}$) the least stable (Lampi et al., 1999). The same observation was made when the aforementioned phytosterols were heated in tripalmitin (Lampi et al., 2000).

Conjugation of the parent sterol also affected its oxidative stability. Esterification with RSO fatty acids made sitosterol and campesterol more reactive than free sterols in a saturated lipid matrix during prolonged heating at 100°C. However, free sterols were slightly more reactive at 180°C. It seemed that the readily oxidizable unsaturated fatty acid moieties in sterol ester molecules started to oxidize during prolonged heating at 100°C, also increasing the oxidation of the sterol moieties. At 180°C, the high temperature also forced the free phytosterols to oxidize, but in the case of steryl

esters, the higher susceptibility of the unsaturated fatty acid moiety to oxidation protected the sterol moiety from reacting. As in the case of free phytosterols and stanols, we noted that the oxidative stability of phytostanols in ester form was greater than that of phytosterol esters, and furthermore, no marked differences were observed between free and esterified stanols. Even when esterified with unsaturated fatty acids, stanols remain stable.

Although sterol ester oxidation has been relatively little studied within the sterol ester molecule the researchers have speculated that a physical barrier or a steric hindrance to oxidative attack exist such that oxygen proceeds to the fatty acid first, slowing the attack on the sterol moiety (Oehrl et al., 2001). Oxidation of esters may proceed via an intramolecular oxidation system in which the firstly oxidized fatty acid attacks the sterol within the same molecule (Paniangvait et al., 1995). Different intramolecular interactions thus provide the most probable explanation for differences observed in oxidative behavior of phytosterol ester molecules at variable temperatures. Based on our unpublished observations and studies conducted by Korahani et al. (1982), the degree of unsaturation of the fatty acid moiety in the sterol ester molecule may also have a significant effect on oxidation of the sterol moiety. When cholesterol acetate, oleate, stearate, linoleate, and linolenate were oxidized in a solid state at 100°C, the initial rate of oxidation of the cholesterol moieties was greater for unsaturated esters than for saturated ones; but after 10 h of heating, the percentage of oxidized cholesterol was higher for saturated esters (Korahani et al., 1982). Further, the more unsaturated the fatty acid moiety in the cholesterol ester molecule in the tripalmitin matrix, the more it accelerated sterol oxidation at 100°C and retarded it at 180°C as compared with free cholesterol (Soupas et al., unpublished data).

The most surprising results were obtained when phytosterols were heated at variable temperatures in different lipid matrices. Our first finding was in accordance with the lipid autoxidation literature; temperature had a marked accelerating effect on the oxidation of phytosterols. It is a well-known fact that increasing temperature produces a greater concentration of free radicals available for the initiation and propagation of lipid autoxidation (Lundberg, 1962). Moreover, a slight increase in the phytostanol oxide contents was observed. As stated in the literature, saturated lipid compounds also undergo slow oxidation, particularly at or above 100°C (Swern, 1961). We then noticed that oxidation of lipid matrix and sterol was coupled in such a way that once the conditions favored lipid matrix oxidation, the oxidative process also spread to the sterol compounds. Closer examination revealed that the situation was, in fact, far more complex.

Interactions observed between different lipid matrices (unsaturated vs. saturated) and temperatures resulted in the hypothesis that at high temperatures (>140°C) the unsaturated lipid matrix itself is more readily oxidizing than the saturated one, thus protecting sterols from reacting. In the saturated lipid matrix, the high temperature forced the more reactive lipid components, sterols, to react. This order was reversed at lower temperatures (<140°C), where the pro-oxidative effect of temperature is

lower. Sterols reacted more rapidly in a matrix which was oxidizing itself, also driving sterols to oxidation reactions. Interestingly, a similar "competition" as that reported above between free and esterified sterols was thus observed here when oxidation of sterols was compared in lipid matrices differing in unsaturation degree. In phytostanol studies, no such interactions were observed; at all temperatures, phytostanol oxidized more in an unsaturated matrix than in a saturated matrix. Since the changes occurring in lipid matrices during phytosterol/stanol oxidation were only studied in a preliminary fashion, they did not offer any clear support for the phenomena observed. However, the slight pro-oxidative effect observed with phytosterol esters with regard to a saturated lipid matrix indicated that complex interactions may indeed take place.

A comparison of our results with those of previous studies is difficult. To date, phytosterol oxidation has been poorly examined, and conditions during oxidation experiments have been quite different, hampering exact comparisons. Evidence does, however, exist that co-oxidation of phytosterols with polyunsaturated fatty acids is likely. For instance, when sitosterol (5%) was heated 2 h at 120°C, 29.9%, 24.9% and 11.1% of sitosterol was found as polar components in sunflower oil, lard, and tristearin, respectively (Yanishlieva-Maslarova and Marinova-Tasheva, 1986). Interestingly, the cited study also reported that although sitosterol oxidized less in a more saturated lipid matrix, its participation in autoxidation was more active than that of saturated matrix. This is in line with our suggestions that in a saturated lipid matrix, temperature may force the more reactive lipid components, sterols, to react.

In a cholesterol oxidation study in which mixtures of cholesterol and tristearin, triolein, trilinolein, or milk fat (1:1) were heated at 130°C for 3 h, triolein was the most destructive, while tristearin was the least destructive matrix as measured via the loss of cholesterol (Kim and Nawar, 1991). In the storage experiment of Ohshima et al. (1993), cholesterol oxidized at a higher rate with fish oil triacylglycerols than in pure form or as a mixture with triolein. These results also suggest that co-oxidation or so-called intermolecular oxidation occurs; hydrogen is extracted from sterol by peroxy or oxy radicals of easily oxidizable neighboring unsaturated fatty acids (Paniangvait et al., 1995).

Some sterol oxidation studies have reported that different interactions between sterols and triacylglycerols may be due to different physical states of these compounds (Yanishlieva and Marinova, 1980; Kim and Nawar, 1991). In lipid oxidation studies, oxidation has been observed to be strongly limited by the solid matter state since the penetration of oxidation is reduced (Holman, 1954). Contrary to this, some authors have proposed that in the solid phase radicals react readily with the substrate, continuing the propagation, whereas in the liquid phase they react readily with other radicals, terminating the oxidative reactions (Labuza, 1971). In our study, at the beginning of the heating experiments, the physical states of rapeseed oil (liquid) and tripalmitin (solid; MP 66°C) as well as free phytosterols (MP 140-170°C) and phytosterol esters (MP 30-40°C) were different.

However, since the heating conditions mostly were rather drastic, the effect of different melting points was considered to be minor.

Significance of the oxidation product profile

Findings on the biological effects of phytosterol oxidation products indicate that these oxides may differ in toxicity (Maguire et al., 2003; Ryan et al., 2005), lymphatic absorption (Grandgirard et al., 1999; Tomoyori et al., 2004), and accumulation in tissues (Grandgirard et al., 2004c). However, no consistent data exist of the order of toxicity of these oxides. Until more information is available on the relationship between different sterol structures and their biological effects, studies on factors affecting their distribution during oxidation are needed.

The main oxides characterized and quantified from sitosterol, campesterol, and stigmasterol, during heating and/or storage experiments here were 7α- and 7β-hydroxysterols, 5α,6α- and 5β,6β-epoxysterols, and 7-ketosterols. Several other secondary oxides were also identified, but mainly only traces of these were present in heated samples. During heating the changes in proportions of the main oxides seemed to be associated with the phase of oxidation; 7-ketosterols accumulated when oxidation was regarded to be in the lag phase, while in the dynamic phase the major sterols were 5,6-epoxysterols and 7-hydroxysterols. Reaction pathways were thus not uniform under these conditions. In the absence of kinetic studies in this field, a more comprehensive evaluation of the courses of these reactions is too complex to be carried out here. Furthermore, the observations that i) changes in product profiles were comparable for free and esterified phytosterols other than their extent being different and ii) changes in product profiles differed between unsaturated and saturated matrices at different temperatures despite the overall extent of oxidation being similar, made the evaluation of the reaction courses even more complicated.

Based on one kinetic study conducted on allylic C-7 oxidation of cholesterol, it can be postulated that the product profile depends on the rate constants between such reactions as formation of 7-hydroperoxysterol, reduction and dehydration of 7-hydroperoxysterols, dehydrogenation of 7-hydroxysterols, and epoxidation of the intact sterol (Chien et al., 1998). The highest rate constant at 150°C was observed for 7-hydroperoxide formation, followed by epoxidation, dehydration, reduction and dehydrogenation (Chien et al., 1998). At elevated temperatures, in the absence of water, the decomposition of 7-hydroperoxides has been anticipated to prefer the formation of 7-ketosterols to that of 7-hydroxysterols (Park and Addis, 1986). Interestingly, in a study in which cholesterol was oxidized with sardine oil triacylglycerols for 39 days at 25°C, the following observation was made: during the lag phase (induction period) measured for triacylglycerols, the main oxide was 7-ketosterol, but as the dynamic period started, 7-hydroxy- and 5,6-epoxysterols accumulated (Li et al., 1994). Compared with our study, although the reaction temperature was

totally different, a corresponding connection between the phase of oxidation and a change in oxide proportions was observed.

As a whole, the oxidation products formed from phytosterols follow the rules generally observed in lipid oxidation. Studies on lipid oxidation have revealed that a complex mixture of secondary oxidation products is formed during the decomposition reactions of lipid hydroperoxides, although the exact mechanisms of the formation and the kinetic and thermodynamic factors governing their distribution are not well understood. In most cases, several different pathways seem to be active, with one or more predominating (Gardner, 1987). These pathways lead to the formation of monomeric, polymeric, and/or volatile oxidation products, the monomeric products including such functional groups (hydroxyl, ketone, and epoxy groups) as those investigated here.

The product profile of quantified sitostanol oxides, 7α-hydroxystanols, and 6α-hydroxystanols, as well as the unidentified oxide of "RRT 1.779" also changed during heating, although the overall oxidation remained low in all conditions examined. At the beginning of heating, the main oxide was the unidentified oxide of "RRT 1.779", but as the heating proceeded, its proportion decreased. In our study, systematic oxidation experiments with phytostanols were conducted for the first time. No other information is available on this topic, and thus, no comparison could be made between our results and literature. Noteworthy also is that sitostanol oxides investigated during this study were only tentatively identified. A more conclusive identification of these oxides requires complementary analytical techniques to those used here.

Formation of other oxidation products

The formation of quantified secondary oxidation products did not explain all of the losses observed in the initial phytosterol contents whether they were in free or esterified form. The formation of this "gap" was also noticed in phytostanol oxidation studies. Closer examination revealed that the "gap" was more significant at higher (180°C) than at lower (100°C) temperatures and for free phytosterols and stanols than for esterified sterols/stanols. These "gaps" indicate that dimers and polymers or other oxidation products were also formed during heating. As described above, several different pathways can be active during lipid oxidation, leading to the formation of not only monomeric but also polymeric oxidation products (Gardner, 1987).

With regard to phytostanol oxidation, the formation of the "gap" revealed a new interesting aspect of oxidative behavior of stanol compounds; although the formation of secondary oxides seemed to be low even during rather drastic heat treatments, significant losses in initial phytostanol contents still occurred. This finding revealed that in order to understand the overall deterioration of both phytostanol and phytosterol compounds, the stanol/sterol loss also needs to be studied. Noteworthy, however, is that although the highest phytosterol/stanol loss in this study was observed for free

sitostanol (41.1%), it was only measured after intense heating (180°C/6 h). No exact comparison could be made between phytosterols and stanols since the end point in sterol heating studies conducted at the same temperature was 3 h.

To understand the "gap", we have conducted preliminary studies using SPE and HPSEC to fractionate some thermo-oxidized samples, and then used high-performance liquid chromatography-mass spectrometry (HPLC-MS) to identify the products in these fractions. As expected, a higher molecular weight material than native sterols or sterol oxides was determined in all SPE and HPSEC fractions, indicating the presence of polar and less polar di- and oligomers of both sterols and sterol oxides (Soupas et al., unpublished data). The "gap" has also been noticed in some cholesterol oxidation studies; when pure cholesterol was heated at 180°C for 1 h, a fraction that totalled 21.1% of the initial cholesterol represented material of a higher molecular weight than the substrate as measured by size exclusion chromatography (Kim and Nawar, 1993). In another study, under the same heating conditions, the total oxides detected accounted for only 30% of the cholesterol loss. The authors speculated that a major portion of the cholesterol decomposition had thus occurred via a different pathway such as, for instance, polymerization (Nawar et al., 1991).

Based on the polymerization process of triacylglycerols, dimerization and polymerization of sterols can, indeed, be expected during heating at high temperatures. As observed during heating of polyunsaturated triacylglycerols, both unpolar nonoxygenated and polar oxygenated dimers and oligomers can be produced. The former can be referred to as "thermal oligomers" and the latter as "oxidative oligomers" (Frankel, 1998). In cholesterol oxidation studies, performed by Lercker and Rodriguez-Estrada (2002), the authors observed that when heating cholesterol above 170°C, a condensation product, 3,3'-cholesterol ether was produced. When further considering the fate of phytosterols/stanols during heating, it is also possible that the "gap" partly originates from the formation of sterol dehydration (sterenes) or dehydrogenation (mono-oxygenated sterols with no hydroxyl group but with mono-, di-, or triunsaturation) products (Bortolomeazzi et al., 2000; Dutta and Savage, 2002a). We also thought that the "gap" may have been partially due to changes in the lipid matrices at high temperatures, which might have led to the formation of structures that bind sterols, making them analytically less available.

6.3 Oxidative stability of phytosterols during food processing and storage

About food applications

Food model studies gave simplified but multilevel insights into oxidative behavior of phytosterols and stanols in common food lipids. As fat usually represents a minor component in many foods, the other food constituents may have a strong influence on the rate and mechanism of oxidation (Labuza, 1971). We therefore evaluated oxidative stability of phytosterols and stanols in enriched

foods as well. Food applications studied covered a range of commercially available phytosterol/stanol ingredients, i.e. phytosterol and stanol esters, wood-based free phytosterols, and microcrystalline phytosterol suspensions in different fats and oils. Processes that were applied during the production of enriched foods included heat treatments that are generally encountered during food processing as well as such critical processes with regard to oxidation as spray-drying, pan-frying, and UHT-type heating. After production, heat-treated foods were subjected to long-term storage at different temperatures since food types studied here are often stored for prolonged times. Different structures of multiphase foods, including powders and emulsions, were also investigated, as were various food lipids (rapeseed oil, butter oil, and rapeseed oil-based liquid margarine). All foods evaluated were enriched with phytosterol/stanol in amounts encountered in real phytosterol-enriched foods.

Food processing and storage conditions with minor effects on phytosterol oxidation

Despite that phytosterols and stanols in microcrystalline suspensions, milks, and milk powders were subjected to many oxidation-affecting factors, i.e. surfaces, nature of lipid compounds, proximity of other food components, and presence of water (Labuza, 1971), their oxidative stability remained good. Based on observations in food model studies and the initial oxidation levels quantified, the oxidative changes measured during food processes applied were considered to be as insignificant. In addition, phytosterols and stanols were stable during storage, although long-term storage subsequent to heat treatments can be regarded as a risk since storage is linked to oxidation during processing, with a higher initial oxidation level accelerating oxidation during storage (Lingnert, 1992). The amounts of phytosterol oxides found after processing and storage were even lower than the initial phytosterol oxide contents (0.1-0.3%) measured in food model studies. Generally, this indicates that there is a good possibility that more oxides are derived from raw materials used in production of enriched foods than from processes applied during enrichment. This interpretation was also proposed by Grandgirard et al. (2004a) and Conchillo et al. (2005), after noting that approximately 0.07-0.08% of phytosterols were oxidized in a commercial phytosterol ester-enriched spread.

Our overall conclusion was that exposure to phytosterol oxides from phytosterol/stanol-enriched heat-treated milks, phytosterol-enriched whole milk powders, or microcrystalline phytosterol suspensions in different fats and oils is unlikely to represent an important source of phytosterol/stanol oxides. Furthermore, considering the combination of heating and subsequent storage, we believe that the deterioration of sensory quality of these products occurs long before any significant quantities of phytosterol oxides are present. Thus, processing comprising heat treatments and subsequent long-term cold storage did not seem to be a limiting factor for the oxidative stability of the foods investigated. It is noteworthy, however, that all storage experiments in the study were conducted in the dark. Light-induced lipid oxidation may be of importance in foods (Lingnert,

1992). Phytosterol oxidation, during storage in the dark (foil bags) was also studied by Lee et al. (1985), who found sitosterol oxides in potato chips after 95 days of storage at 40°C. At a lower temperature, 23°C, a storage of 150 days produced no detectable amounts of oxides (the detection limit was not specified). The authors concluded that in foil-bagged chips, sitosterol oxidation occurs at a point far beyond the overall product acceptability (Lee et al., 1985).

Moreover, it should be emphasized that the observed changes in phytosterols were lower than predicted. Firstly, microcrystalline phytosterol suspensions in fats and oils with different fatty acid compositions could have differed in stability during long-term storage. A similar active participation of an unsaturated lipid matrix in phytosterol oxidation as that observed in food model studies was not, however, seen. Lowering the storage temperature (4°C) was thus a way to improve the stability of phytosterols in different lipid matrices. Secondly, we thought that whole milk powder, because of its large surface area, could have been more susceptible to oxidation as an interfacial phenomenon. Generally, due to greater oxygen diffusion (Labuza, 1971) and closer contact of oxidizable lipids and metals as pro-oxidants (Frankel, 1998), powders are considered to be sensitive to oxidation. Thirdly, we anticipated greater changes in enriched milks since milk as oil-in-water emulsion can be susceptible to oxidation due to the large interfacial area between oil and water. In emulsions, lipid oxidation can be markedly influenced by the interaction between lipid hydroperoxides located at the emulsion droplet interface and transition metals originating in the aqueous phase (Frankel, 1998).

Finally, it is noteworthy that despite the good oxidative stability of the phytosterols observed in our storage experiments, the risk of sterol oxidation always exists due to the presence of the above-mentioned oxidation accelerating factors. In cholesterol oxidation studies, many of the conditions presented here have been shown to enhance sterol oxidation, leading to the general conclusion that oxides are formed in most processed products containing sterol and the prevention of this reaction is difficult unless the products are totally sterol-free (Tai et al., 2000).

Food processes with moderate effects on phytosterol oxidation

Interestingly, phytosterols were not stable in all food applications and processes tested. Pan-frying at high temperatures (160-200°C) seemed to induce phytosterol oxidation, although phytostanols remained stable. Based on food model studies, the finding that the phytosterol oxidation (up to 5.1%) observed during 5-10 min of pan-frying can only be reached after 0.5-2 h of heating in an oven at 180°C indicates than pan-frying is, indeed, a rather deteriorative process. Pan-frying may induce phytosterol oxidation because of its high temperature and large surface-to-volume ratio, which allows high oxygen adsorption per unit of frying oil (Usuki et al., 1980). When, for instance, a thin film of low-linolenic acid soybean oil was fried in a Teflon pan at 180°C, the polymer content exceeded 20% already after 10 min of frying (Soheili et al., 2002). In the present study, interactions

between the iron pan and the thin film of oil could also have accelerated oxidation – a phenomenon observed by Takaoka and Kobayashi (1986) as well.

The extents of deteriorative reactions were determined by frying temperature and time, but also by unsaturation degree and water content of the lipid matrix and by structure of the phytosterol compound added. Interestingly, most phenomena discovered during food model studies were also observed in frying. Important findings made were i) the higher the temperature and the longer the reaction time, the more phytosterols oxidized, ii) the percentage of phytosterol oxidation at high temperatures increased when a saturated lipid matrix was used, and iii) phytosterols oxidized slightly more in free than in esterified form at high temperatures.

New aspects of phytosterol oxidation were also identified during pan-frying. Firstly, natural phytosterols in rapeseed oil were found to oxidize similarly to the added phytosterols. This indicates that added phytosterols can be bound by the lipid matrix in the same manner as indigenous phytosterols, and thus, similarly exposed to oxidation reactions. Secondly, the presence of water in the lipid matrix was revealed to play an important role in sterol oxidation; phytosterols oxidized more in the presence of water (RSO vs. RSO-based liquid margarine). This finding is most probably related to the reaction of water with frying oil, resulting in the development of free fatty acids, which, in turn, accelerates the oxidation of phytosterols. Free fatty acids are known to be more rapidly oxidizable and to promote thermal oxidation by solubilizing metal catalysts (Frankel, 1998). Considering the hydrolysis of frying oil in the presence of water, the question also arose of the fate of phytosterol/stanol esters during frying. Breakage of ester bonds may have occurred but no studies were conducted to resolve the extent of possible hydrolytic reactions. Generally, these reactions are regarded as less prevalent than oxidation and polymerization, at least in deep-frying processes (Kochhar and Gertz, 2004).

To clarify the overall changes occurring during pan-frying, the deterioration of lipid matrices was also investigated in terms of polymerization. Pan-frying caused a moderate degree (up to 4.8%) of lipid matrix polymerization. Depending on the lipid matrix and the frying temperature, changes in lipid matrices were either more drastic or less drastic than in phytosterols. Furthermore, interesting interactions were again noticed between sterols, matrices, and temperatures, e.g. at 180°C, phytosterol oxidation seemed to enhance polymerization of liquid margarine, but at 160°C, inhibition of rapeseed oil polymerization was observed. The slight oxidation-promoting and -inhibiting effects of phytosterols require, however, confirmation by more detailed studies.

In summarizing this pan-frying study, it should be noticed that only enriched oils were fried here, and thus, neither the influence of food on frying oil stability nor the impact of frying oil on food quality was evaluated. Foods can change the composition and stability of the frying oil, and this oil can then be absorbed by foods in amounts varying from 5% to 40% (Frankel, 1998). Already, in the

1980s, the partition of phytosterol oxides, formed in plant-derived oils during frying, into foods was reported (Finocchiaro and Richardson, 1983). Later, Dutta et al. (1996) determined phytosterols and phytosterol oxides in fried potato products and found considerably higher amounts of sterol oxides in French fries prepared in a RSO/palm oil blend than those fried in sunflower or high-oleic sunflower oil. They suggested that differences observed in oxide contents of French fries originated from different phytosterol contents in the frying oils oils, i.e. the more phytosterols in the frying medium, the more oxidized phytosterols in the fried food item. The necessity of using good-quality frying media is thus obvious making the possible future marketing of phytosterol-enriched oils challenging.

New findings in oxide profiles

The spectrum of identified oxides in food application studies was broader than in food model studies. Generally, the spectrum and the amount of oxides formed were strongly dependent on the phytosterol composition in the phytosterol preparations used in enrichment. Since the overall oxidative reactions observed in food applications, other than pan-frying, were so minor, the changes observed in oxide proportions were not as clear as in food model studies. In pan-frying, however, the same trend as in model system studies was noted; at the beginning of heating, the main oxides were 7-ketosterols, but as frying proceeded, the proportions of 7-hydroxy- and 5,6-epoxysterols increased. In the case of phytostanol-enriched samples, no clear comparison could be made with food model studies since the oxides quantified were partly different.

Interesting findings during these food application studies were the high initial amounts of 6-ketositostanol in samples enriched with wood-based phytosterols and 6α-hydroxysitostanol in samples enriched with phytostanol esters. Although the formation of, at least, 6α-hydroxysitostanol was established during the oxidation experiments also, the both oxides mentioned could have been derived from the raw materials used, as their initial contents in relation to other oxides were high. Previous studies have, indeed, shown that 6-hydroxysitostanols are present in refined rapeseed oils (Lambelet et al., 2003). 6-ketostanols are speculated to form by rearrangement of 5,6-epoxysterols (Yanishlieva-Maslarova et al., 1982; Conchillo et al., 2005).

7 CONCLUSIONS

Scientific data to support the safety evaluation process of phytosterol/stanol-enriched functional foods in terms of oxidative stability was produced. The food model studies conducted gave simplified but multilevel insights into oxidative behavior of phytosterols and stanols in common food lipids. To further extend understanding in this field, food applications covering a range of commercially available phytosterol/stanol ingredients, different food processing methods, and different structures in multiphase foods were investigated. Our results contribute to the understanding of how phytosterol/stanol oxidation, as a potential risk for human health, proceeds in foods and reveal the critical factors affecting oxidation.

As no official method exists to determine phytosterol/stanol oxidation products in foods, a new method for their characterization and quantification in complex food matrices was taken into use. The GC-MS method developed offers a powerful tool for measuring phytosterol and phytostanol oxidation products in foods. Furthermore, the indirectly constructed GC-MS calibration, which relies on oxides produced by thermo-oxidation and quantified by GC-FID, was concluded to be a suitable compromise in the present situation, where no commercial pure standards exist for phytosterol/stanol oxidation products. Overall, the new GC-MS method showed good repeatability and acceptable accuracy compared with the more routinely used GC-FID method, particularly, in view of low oxidation product amounts and the complexity of food materials.

The data obtained in food model studies revealed that the critical factors for the formation and distribution of the main secondary oxidation products were sterol structure, reaction temperature, reaction time, and lipid matrix composition. In all conditions studied, phytostanols as saturated compounds were more stable than phytosterols. Moreover, esterification made phytosterols more reactive at low temperatures than free phytosterols, while free phytosterols were more reactive at high temperatures than esterified phytosterols. Generally, oxidation reactions were more significant at temperatures above 100°C. At lower temperatures, the significance of these reactions increased with increasing reaction time. The effect of lipid matrix composition was surprisingly dependent on temperature; at temperatures above 140°C, phytosterols were more stable in an unsaturated lipid matrix, whereas below 140°C they were more stable in a saturated lipid matrix. At 140°C, phytosterols oxidized at the same rate in both matrices. Regardless of temperature, phytostanols oxidized more in an unsaturated lipid matrix.

The oxidation product profiles of both phytosterols (hydroxyl, epoxy, and ketone compounds) and stanols (mainly hydroxyl compounds) changed during heating at different temperatures. Generally, the changes in proportions seemed to be associated with the phase of overall oxidation. 7-ketophytosterols accumulated when oxidation had not yet reached the dynamic state. Once this state was attained, the major products were 5,6-epoxyphytosterols and 7-hydroxyphytosterols. The

changes observed in phytostanol oxide proportions were not as informative since all of the stanol oxides quantified represented hydroxyl compounds. Interestingly, the formation of these secondary oxidation products did not account for all phytosterol/stanol losses observed during heating. This indicated the presence of dimeric and oligomeric oxidation products, especially when free phytosterols and stanols were heated at high temperatures. This finding was significant considering, in particular, the oxidation of phytostanol compounds; although the formation of secondary oxidation products seemed to be low, significant losses in initial stanol contents occurred. This phenomenon was, however, valid only in intense heating conditions.

Commercially available phytosterol/stanol ingredients (phytosterol and stanol esters, wood-based free phytosterols, and microcrystalline phytosterol suspensions) were stable in normal food processing conditions, including spray-drying and UHT-type heating. These ingredients also proved to be stable during prolonged storage even at slightly elevated temperatures. Pan-frying, however, seemed to induce phytosterol oxidation. In conjunction with other observations in this study, pan-frying was classified as a rather deteriorative process with regard to phytosterol oxidation.

Our findings indicate that although phytosterols and stanols are stable under normal food processing and storage conditions, attention should be paid to their use in frying purposes. Complex interactions between other food constituents also suggest that when new phytosterol-enriched novel foods are developed, their oxidative stability must be established. The results presented here will assist when choosing conditions in which phytosterol/stanol enrichment can be considered to be safe.

Future research should proceed towards more mechanistic studies to further resolve the diverse pathways through which phytosterols and stanols can oxidize. The most interesting compounds will be esterified phytosterols and stanols and their oxidative behavior in the presence of other food lipids. Continued improvement of analytical methods remains a challenge.

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